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ARTICLE

Effect of sugars on production of β-sitosterol from in vitro callus culture of *Boerhaavia diffusa* L.

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ABSTRACT The establishment of friable callus was achieved on Murashige and Skoog (MS) medium fortified with 18 µM of 2,4-dichlorophenoxyacetic acid in combination with 2 µM of 6-benzylaminopurine (BAP). The effect of different sugars such as 3% sucrose, 3% glucose and 3% galactose on the growth of callus tissue and β-sitosterol accumulation was tested. Quantification of β-sitosterol was carried out with the help of high performance liquid chromatography. High growth of callus tissue was observed on MS medium, containing sucrose and glucose as carbon sources and maximum β-sitosterol content was observed in 60 days old callus tissue with 3% glucose as carbon source.
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KEY WORDS

Boerhaavia diffusa
β-sitosterol
callus
glucose
sucrose

Abbreviations

MS - Murashige and Skoog, 2,4-D - (2,4-dichlorophenoxy acetic acid), TLC - Thin Layer Chromatography.

Introduction

Boerhaavia diffusa is a creeping, perennial herbaceous plant that belongs to the family Nyctaginaceae (Four o'clock Family). Although it is native to India and Brazil, but it has been traditionally used as a medicine in many different parts of the world (Patil and Bhalsing 2016). Out of the 40 species of the genus *Boerhaavia*, five were found in India (*B. diffusa*, *B. chinensis*, *B. erecta*, *B. rependa*, and *B. rubicund*) (Chopra et al. 1969). In Ayurvedic and Unani medicine, *B. diffusa* L plant is mentioned to cure 22 different types of ailments. In Brazilian pharmacopeia, 23 traditional uses have been described for this plant, while in Africa and Middle East the plant is prescribed for 14 ailments (Apu et al. 2012). In many other traditional medicines, root and aerial parts of this plant were reported for the treatment of diabetes, jaundice, infectious diseases and urinary problems (Patil and Bhalsing 2016).

B. diffusa is among the 46 medicinal plant species in high trade sourced mainly from the wastelands (Ved and Goraya 2010). Beside this, the plant is an exclusive source or used in polyherbal formulations, including Himalaya, Dibecon, Dabur Chyawanprash, etc.

The plant possesses coccineone B, coccineone E, boeravinone C, boerhavinone D, boerhavinone F, boerhavinone G, boerhavinone H, boerhavinone I, boerhavinone J (Borelli et al. 2010), boerhavinone P, boerhavinone Q, boerhavinone R, boerhavinone S, boerhavinone M (Bairwa et al. 2013a and b), β-sitosterol (Patil and Bhalsing 2016), and eupalitin (Pandey et al. 2010).

Phytosterols have many useful effects on human health,which has increased demand and consumption of phytosterol-enriched foods. More than 40 patents on phytosterol products and more than 15 commercial plant sterol products were incorporated in the market (Ana et al. 2013). Among 200 different types of phytosterols, β-sitosterol (65%) and stigmasterol (30%) are most widely consumed. This requires huge quantity of plant materials for extractions, which can have serious impact on the environment (Lagarda et al. 2006; Ana et al. 2013). Also, due to the effects of different environmental conditions like pollutants and pathogens, the naturally grown plants used for drug preparation can affect productivity (Nikam et al. 2009). The commercial bulk of *B. diffusa* possesses a heterogeneous population, which results in poor quality roots and biomass (Saini et al. 2011; Patil and Bhalsing 2015). Plant tissue culture technique can serve as an ideal system to circumvent the above said problems. Moreover, a uniform quality of secondary metabolites can be obtained from tissue culture technology, due to clones or less genetic variations. The β-sitosterol was isolated from tissue cultures of plants such as *Balanites aegyptiaca* (Bidawat 2006), *Citullus colocynthis* (Meena et al. 2011) and

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Terminalia chebula (Archana et al. 2011). There is continued research on finding the new sources. There are few reports of tissue cultures including *in vitro* regeneration and callus induction in *B. diffusa* (Patil and Bhalsing 2015; Jain et al. 2003; Wesely et al. 2011). Srivastava et al. (1995) reported alkaloid content of *in vitro* regenerated roots of *Boerhaavia diffusa*. However, there seems no any report of isolation of secondary metabolite from *in vitro* callus culture of *B. diffusa* L. Here, we established the protocol for isolation and estimation of β-sitosterol from friable callus of *B. diffusa* L and further demonstrated the effect of different sugars on accumulation of β-sitosterol in callus cultures.

Materials and Methods

Plant material and chemicals

The plants of *B. diffusa* were obtained from the campus of North Maharashtra University, Jalgaon and used as a source of explants for experiment. The plant specimen was authenticated and deposited at the Botanical Survey of India Regional Office, Western Circle, Pune, India (specimen voucher number KSP-01). Standard β-sitosterol was purchased from Sigma Aldrich, India. All other solvents used for extraction and chromatography conditions were HPLC-grade and obtained from Fisher Scientific (PA, USA).

Surface sterilization, media preparation, establishment and maintenance of callus culture

The mature leaf explants of *B. diffusa* were thoroughly washed under running tap water for 10-15 min accompanied with 2-3 drops of Tween 20 to ensure removal of dirt. Then the explants were treated with 0.4 g/l of antifungal agent, *i.e.* bavistin (BASF, India) and washed 3-4 times with sterile distilled water. These antifungal treated explants were surface sterilized with 0.1% $HgCl_2$ for 2 min and washed 3-4 times with sterile distilled to remove any traces of $HgCl_2$. All these surface sterilization steps were carried out aseptically in laminar airflow hood (Laphosp™). MS basal medium was used for the experimental part with 3% (w/v) sucrose as carbon source and 0.8% (w/v) agar. The pH was adjusted to 5.6±0.2 using 1 N HCl or 1 N NaOH before adding 0.8% (w/v) of agar. The medium was autoclaved under 105 kPa at a temperature of 121 °C for 15 min. All growth regulators were filter-sterilized (0.22 µm, Millipore) and added after the autoclave. The above trimmed surface sterilized leaf segments of *B. diffusa* L were inoculated aseptically on this sterile MS medium, fortified with 18 µM 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 2 µM of 6-benzylaminopurine (BAP) for callus induction. Cultures were incubated at 25±2 °C in 16/8 h photoperiod with light intensity of 2000 lux provided by white, fluorescent tubes (Philips™) and 55±5% relative humidity.

Effect of different sugars on callus induction and accumulation of β-sitosterol

Different sugars such as 3% glucose and 3% galactose were incorporated in MS medium, supplemented with 18 µM 2,4-D+2 µM BAP for callus induction and β-sitosterol accumulation. A negative control was set in the experiments, which do not contain any carbon source and 3% sucrose served as a positive control.

Determination of Growth index

The proliferated calli were subcultured at 30 days intervals on fresh culture medium. Growth index was determined after initial subculturing for 20, 45, 60, and 80 days old calli using following formula.

$$\text{Growth Index} = \frac{\text{Final fresh weight of callus} - \text{Initial fresh weight of callus}}{\text{Initial fresh weight of callus}}$$

Extraction of β-sitosterol from in vivo plant parts and callus cultures

In vivo plant parts and harvested calli were air dried and powdered. Each powdered material (2 g) was hydrolysed with 25 ml of 30% (v/v) hydrochloric acid for 4 h in a water bath at 60 °C. The hydrolysed extract was filtered and washed with distilled water through Whatman filter paper no. 1 until the filtrate reaches around pH 7. The obtained residue was dried at 60 °C for 8 h and Soxhlet extracted with benzene (200 ml) for 24 h. The benzene extracts were dried and reconstituted in chloroform and used for further analysis (Archana et al. 2011; Meena et al. 2011).

Thin layer chromatography(TLC)

The crude extracts along with a standard reference sample of sterols (β-sitosterol) were applied separately on silica gel 'G' (Merck Ltd., Mumbai, India) coated plates (20 × 20 cm; wet thickness 0.2-0.4 mm), which were activated at ~60 °C in an oven for 30 min and brought to room temperature before use. The plates were developed in organic solvent mixtures of hexane:acetone (8:2, V/V). The developed plates were air dried, sprayed with 50% sulphuric acid and subsequently heated at 100 °C for 10 min (Meena et al. 2011).

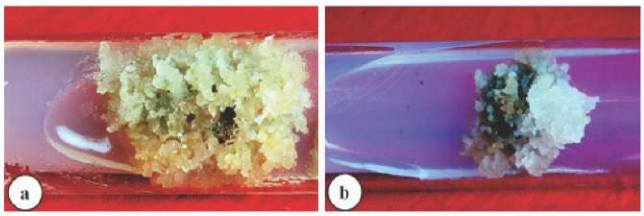

Figure 1. Callus formation on MS medium supplemented with 18 µM of 2,4-D+2 µM BAP. (a) callus obtained on 3% sucrose (b) callus obtained on 3% glucose.

Figure 2. The β-sitosterol detection in leaf derived callus extract of *Boerhaavia diffusa* L TLC plate. C=callus extract; S=standard (β-sitosterol).

HPLC analysis

Different concentrations of standard β-sitosterol were prepared and subjected to HPLC to obtain the calibration curve of β-sitosterol. The purified sample of β-sitosterol from callus tissue and *in vivo* parts of plant *viz*., leaves, root and stem were subjected to HPLC for obtaining the chromatograms. HPLC analysis was carried out on a Young Lin (S.K) Gradientcratic System provided with an autosampler (injection volume 20 µl). The separation was made on a reversed phase C18 column (Agilent Technologies, USA) (4.6 x 150 mm) of 5 µm packed size particles in conjunction with Autochro-3000 software. The HPLC was equipped with SP 930 D isocratic pump and UV 730 D detector (Young Lin, Korea). The detection wavelength was set at 220 nm. The samples were eluted at a flow rate of 0.5 ml/min using mobile phase acetonitrile:water (90:10, V/V) (mix pH 3).

Statistical analysis

Each experiment was carried out in triplicates and data from all experiments were used to calculate the mean and standard deviations in Microsoft Excel 2013.

Results and Discussion

Growth index and morphology of callus

Here, we have established the friable callus on MS medium supplemented with 18 µM of 2,4-D + 2 µM BAP (Fig. 1). A pale yellow, friable callus was obtained on MS medium supplemented with 3% sucrose. The well-proliferated calli harvested after 20, 40, 60, and 80 days showed an increase in the Growth index up to 60 days and slightly declined thereafter (Fig. 5). Similar patterns of growth with maximum growth index on 60 days old callus was observed with other sugars incorporated in MS medium. A necrosis with almost no growth was observed in negative control, which was lacking any carbon source.

Effect of different sugars on growth of the callus

The development of callus was observed in each of the sugar *viz*., 3% sucrose, 3% glucose and 3% galactose incorporated in MS medium supplemented with 18 µM of 2,4-D+2 µM BAP. However, in our study, the growth of the callus was found to be high in MS medium with 3% sucrose and 3% glucose as carbon sources as compared to MS medium containing 3% galactose (Fig. 6). A brownish callus with least growth, was observed in medium containing galactose.

Thin layer chromatography

When the developed plates were sprayed with 50% sulphuric acid, the purple colored spot appeared of the callus extracts applied, which are coincided with those of standard compound samples of β-sitosterol. Rf value (0.55) of β-sitosterol isolated from the samples equal to the Rf value of reference compound β-sitosterol (Fig. 2). Similar results were observed by Sen and co-workers (2012) by identifying β-sitosterol in leaves of *Momordica charantia*.

Effect of different ages on β-sitosterol accumulation.

HPLC chromatogram was developed for each *in vivo* plant parts and calli harvested after 20, 40, 60 and 80 days. Quantification was carried out using linear regression equation obtained from the HPLC calibration curve of standard β-sitosterol. HPLC chromatogram of standard β-sitosterol and callus (60 days old) showed the peak of β-sitosterol in Fig. 3 A-C. Among the different plant parts *viz*., roots, stems and leaves of *Boerhaavia diffusa* L, maximum content of

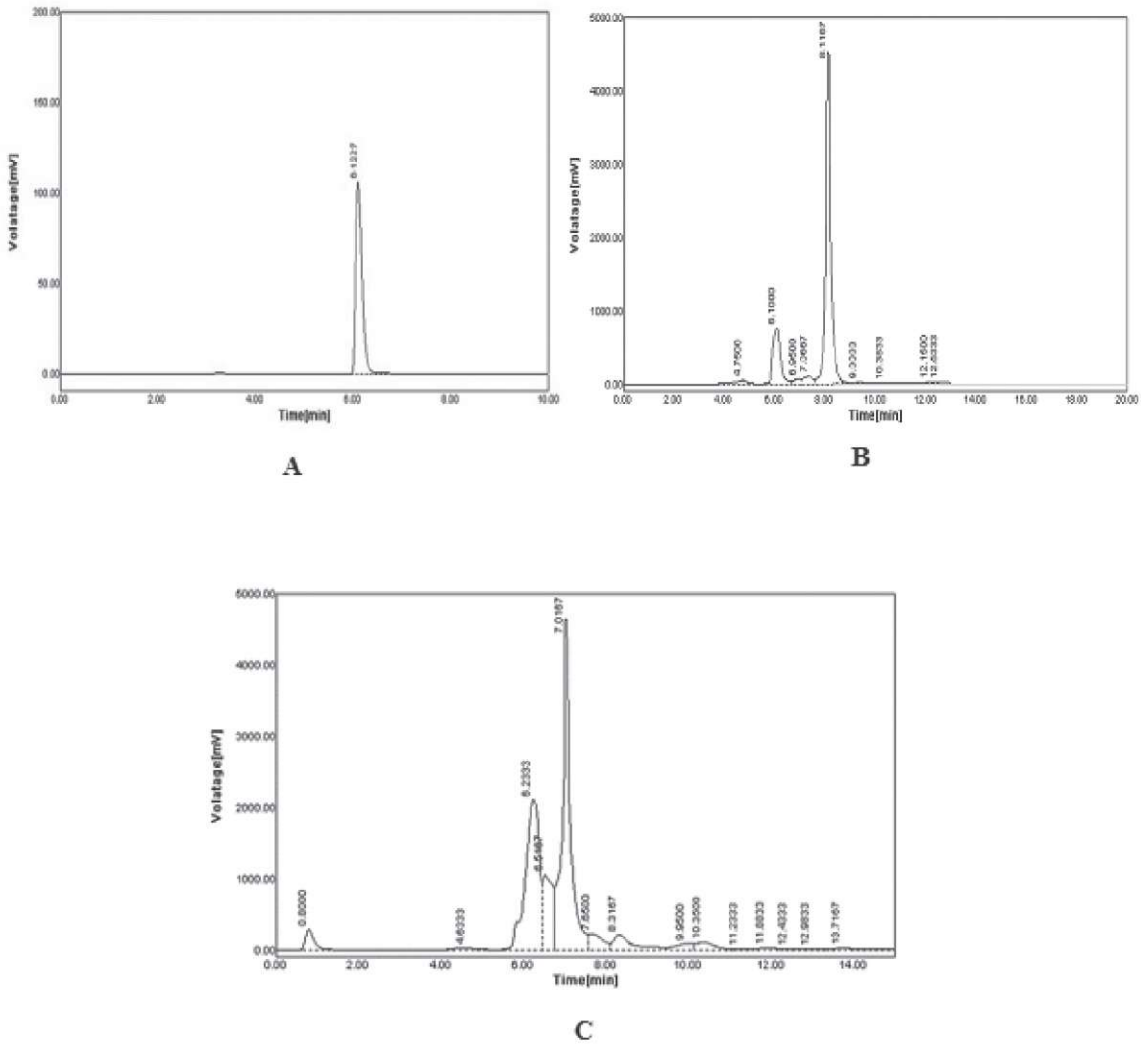

Figure 3. (A) HPLC chromatogram of standard β-sitosterol. (B) HPLC chromatogram of callus extract cultivated with 3% sucrose. (C) HPLC chromatogram of callus extract cultivated with 3% glucose.

β-sitosterol was observed in roots followed by leaves (Fig. 4). However, compared to *in vivo* leaves, the *in vitro* developed leaf derived calli (specifically 60 days old) showed higher accumulation of β-sitosterol (Fig. 4, 5). Chawla and Bansal (2014) also observed more percentage of β-sitosterol in *in vitro* regenerated bark and callus compare to *in vivo* bark of *Helicteres isora* L. They further concluded that phytohormones used for *in vitro* propagated plants, may have favorable role in accumulation of higher β-sitosterol. In our present study, the content of β-sitosterol was increased with an increase in the growth index of callus. Maximum β-sitosterol (456.01±9.2 µg/g dw) was accumulated in 60 days old callus culture, which decreased in the culture collected on 80 days (443.2±11µg/g dw) (Fig. 5). Many workers have also observed the higher percentage of β-sitosterol in 6 weeks and decrease in percentage thereafter (8 weeks) in callus tissue of *Citullus colocynthis* (Meena et al. 2011) and *Terminalia chebula* (Archana et al. 2011). They also observed more accumulation of β-sitosterol in callus culture than their *in vivo* plant parts. Plant hormones such as 2,4-D and BA was also favorably accumulated the harmala alkaloids in calli of *Tribulus terrestris* (Nikam et al. 2009). This suggest that plant growth hormones, beside growth and differentiation, may have influence on accumulation of secondary metabolites. Production of sterols from *in vivo* and *in vitro* tissue culture, their structure, composition, effects, biochemistry, identification and their function has also been reported in several plants since last few years (Clifton et al. 2004). Our study is significant in the establishment of callus as an alternative source of metabolites in *Boerhaavia diffusa* L.

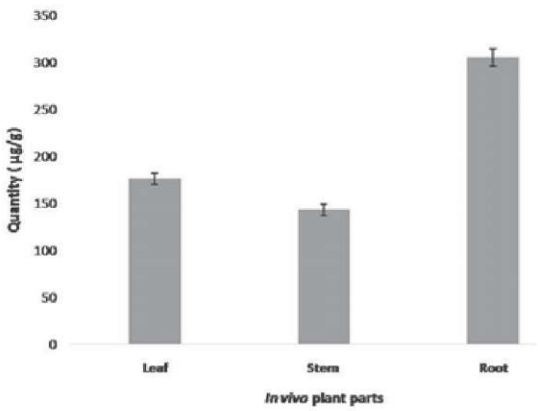

Figure 4. The β-sitosterol content in different *in vivo* plant parts of *Boerhaavia diffusa* L.

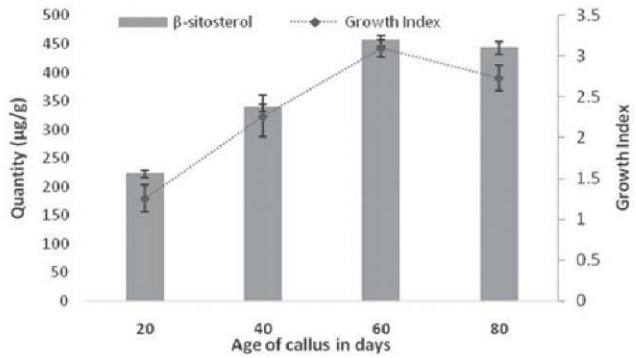

Figure 5. Effect of different ages of callus on accumulation of β-sitosterol in *Boerhaavia diffusa* L. under the influence of 18 µM of 2,4-D+2 µM BAP with 3% sucrose. Bars represent the quantity of β-sitosterol with mean±SD of three independent experiments and line represents the Growth index.

Effect of sugars on β-sitosterol accumulation

Besides the phytohormones, the types of sugar also influenced the production of β-sitosterol. In callus culture produced on MS medium containing 3% glucose as carbon source has profound effect on production of β-sitosterol. The β-sitosterol accumulated in this case was 575.27±15.0 µg/g dw, while least accumulation of β-sitosterol was observed in callus produced on MS medium containing 3% galactose (Fig. 6).

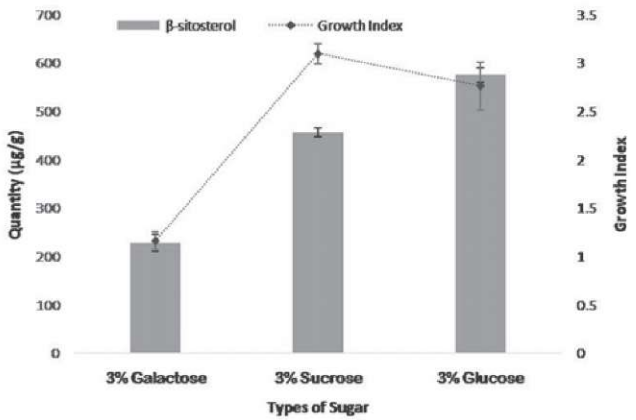

Figure 6. Effect of different sugars on accumulation of β-sitosterol in callus cultures of *Boerhaavia diffusa* L. Bars represent the quantity of β-sitosterol with mean±SD of three independent experiments and line represents the Growth index of 60 days old callus.

Conclusion

Present study concluded that different ages of the callus tissue have influences on the secondary metabolite production. Also, the culture conditions, including phytohormones can also affect the β-sitosterol accumulation. In the present protocol, different sugars have a profound effect on β-sitosterol productions in callus tissue. As there is continual establishment of β-sitosterol potentials in different pharmaceutical drugs, the present protocol is of considerable interest as the β-sitosterol accumulation is found to be more in *in vitro* culture than *in vivo* plant parts.

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ARTICLE

Comparative ontogenetic survey of the essential oil composition in *Origanum vulgare* L., and *Origanum majorana* L.

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ABSTRACT The aim of this study was to evaluate the influences of the different harvest stages on the value and components of the essential oil (EO) of wild oregano (*Origanum vulgare* L.) and European marjoram (*Origanum majorana* L.). Two species were collected during five stages of vegetative and reproductive growth period with four replications. The content of EO in the dried aerial parts was determined by hydro-distillation of herbs, and its constituents determined by gas chromatography (GC) and gas chromatography mass spectrometry (GC/MS). The analysis showed that the amount of EO in *O. majorana* was more than *O. vulgare*. The highest EO content for both species were obtained in the full flowering stage. 78 and 39 components were identified in the EO of *O. vulgare* and *O. majorana*, respectively. The main components were germacrene D, (*trans*)caryophyllene, terpinene-4-ol, and α-terpinene in the EO of *O. vulgare*, and terpinene-4-ol, γ-terpinene, α-terpinene, and α-terpineol in the EO of *O. majorana*. In general, sesquiterpene compounds had maximum amounts in the EO of *O. vulgare* in five stages of growth, especially in the beginning of flowering stage. In contrast, monoterpene compounds had maximum value in the EO of *O. majorana* in the full flowering stage.
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KEY WORDS
germacrene D
harvest stages
marjoram
monoterpene
Origanum
sesquiterpene
terpinene-4-ol

Introduction

Wild oregano (*Origanum vulgare* L.) or Mediterranean oregano is perennial herb with a wide distribution in the Mediterranean region, Irano-Sybryn, and Euro-Sybryn that grows on the rocky slopes in a wide range of altitudes (0-4000 meter) (Kordali 2008). Oregano grows in the Northern areas (Chalous, Rasht and Gorgan), Western (Azerbaijan, Ardebil, and Kurdistan), and Eastern (Ahar and Bojnoord) of Iran (Rechinger 1982). This species growing to 100 cm tall, shoot straight, hairy and reddish-green, oval leaves, dark green and covered with trichome, which has covered the surface of the lower lamina (Afsharypuor et al. 1997). In addition to the traditional use for the treatment of diseases of the stomach, intestines, constipation and respiratory problems (asthma), the aerial parts and the leaves of this species are being used throughout the world as a very pleasant and aromatic spices. Besides, it also has been used as a disinfectant and healer in many applications (Kordali 2008). Prevalence studies have shown the antifungal activity, antibacterial and antioxidant of the species' EO from genus *Origanum* (due to thymol, and carvacrol in them) (Muller 1989; Gouladis et al. 2003). In a study at the National Botanical Garden of Iran, the dominant components of EO in the *O. vulgare* were found to be β-caryophyllene, germacrene D, and (*cis*)sabinene hydrate (Barazandeh 2000). In another relevant study, the main components of EO in the *O. vulgare* ssp. *vulgare* in four regions of Italy were recognized to be β-caryophyllene, thymol, terpinene-4-ol, and (*para*)cymene (Melegari et al. 1995).

Marjoram (*Origanum majorana* L.) is a perennial plant, with opposite leaves, elliptical, toothless and small white flowers covered with 4 rows of whitey bracts. These bracts cause the appearance of globular flowers (Afsharypuor et al. 1997). Biochemical compounds of this species are the EO and tannins. Its essence is obtained by hydro-distillation and has a greenish yellow color and mild odor and taste, 40% terpene especially terpinene, terpinolene and sabinene (Naghdi Badi et al. 2004). Some studies indicate that it has antimicrobial and antifungal properties (Oliveira et al. 2009). This type of majoram has been used in flavoring foods and has some important properties such as calmative effect, energy provider, diuretic, sudatory and stomach tonic (Afsharypuor et al.

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1997). Research at the National Botanical Garden was conducted on this species. So that, sabinee and linalil acetate was the most important compounds of EO (Barazandeh 2000).

So far, many studies have been done on quantitative and qualitative changes in the components of EO and their composition during the period of growth. These changes depending on the type of plant were varied, as in some species the most EO were in pre-flowering stage, the others were in flowering stage and in some of them also the maximum amount of EO identified after flowering stage (Wang et al. 2009). According to previous studies, a scientific research was not performed on quantitative and qualitative changes in EO of oregano and marjoram cultivated in Iran, as yet. Therefore, study the effects of various harvest stages on the quantitative and qualitative of EO in both species was the aim of the present experiment. Additionally, the scientific results can be applied to the use of these herbs in the pharmaceutical and food industries.

Materials and Methods

Plant material and experimental field profile

In this study, seedlings of two species of oregano (*O. vulgare*) and marjoram (*O. majorana*) with registration codes MPISB-259, and MPISB-158, respectively, were prepared from the Germplasm Bank at Medicinal Plants Institute (MPI) of Academic Center for Education, Culture and Research (ACECR). In early April 2013, seedlings were planted at the experimental farm of medicinal plants institute located in the Karaj region for evaluation the influences of the different harvest stages during growth cycle on the value and components of the oregano and marjoram EO in the same climate conditions. The geographical location of the station was 35° 54' 17" N and 50° 53' 7" E with about 1461 m (elevation) altitude above the mean sea level. The soil was loam-silt with 0.071% N, 48.9 mg/kg phosphorous, 33.6 mg/kg potassium, EC 2.71 dS/m, and pH 8.3. During the experimental periods, the mean weekly temperature varied from 24.5 to 43.7 °C in day and 9.1 to 23.5 °C in night for growth season. The average weekly rainfall and weekly relative humidity were 1.4 mm and 50.34%, respectively. Each experimental plot was 5 m long and 2 m wide which prepared after tillage operations. There was 1 m space between the plots and 1.5 meters between the replications. In May, June and July 2013, the aerial parts of plants were collected in five growth stages (two stages in the vegetative phase and three stages in the reproductive phase) between 12:00 and 13:30 P.M. Conditions on the day of collection were clear and sunny for each of the five stages. The harvested plant materials were air-dried in a shaded place at a convenient temperature (22 ± 2 °C) and in an air-flow during 6 days. The samples were transferred to the phytochemical analysis laboratory for measuring the percentage of essential oils and their components. For the sake of accuracy and reducing the possible errors, samples were randomly picked from separate experimental plot with four replications.

Extraction of EO and identification of its components

The content of EO in dried samples was determined by the hydro-distillation of 100 g herb in a Clevenger-type apparatus during 4 h. The oils were dried over anhydrous sodium sulphate and kept at 4 °C until it was analyzed (British Pharmacopoeia 1988; Sefidkon and Abbasi 2006). The extracted essential oils were also identified by gas chromatography (GC) and gas chromatography mass spectrometry (GC/MS) analysis. Besides, the volatile constituents were analyzed using an Agilent instrument coupled with a 6890 mass system equipped with flame ionization detector (FID) equipped with BPX5 capillary columns (30 m × 0.25 mm, i.d. 0.25 µm film thicknesses). The carrier gas was helium, at a flow rate of 0.5 ml per min. The column temperature was gradually increased from 60 to 220 °C with a rate of 2 °C per min for the polar column and from 60 to 240 °C with a rate of 3 °C per min for the non-polar column. The injection volume was set to be 1 µL. The column temperature was considered to be the primary oven temperature 50 °C and 5 minutes at this temperature was stopped, thermal gradient was 3 degrees per minute, increasing the temperature to 240 °C and then at a rate of 15 degrees per minute, increasing the temperature to 300 °C and 3 minutes at this temperature was stopped. Split injector was used at 290 °C in a split mode at a ratio of 1:50. GC/MS analyses were performed using an Agilent 5973 mass spectrogram in the electron impact ionization mode at 70 eV, the mass range was m/z 40-500. Injector temperature was 220 °C. The identity of the components was assigned by comparing their Kováts retention indices (KI) relative to C_8-C_{32} n-alkanes (Sigma Chemical Co., St. Louis, MO), obtained on non-polar DB-5 column with those provided in literature. Tentative identification of the compounds based on the comparison of their relative retention time and mass spectra with those of the NIST-98 and Wiley-275 library data of the GC/MS system and the literature data (Adams 2007) was carried out. Each extraction and the compound percentages were replicated four times.

Statistical analysis

All the obtained data were used in statistical analysis (Proc GLM) based on a one-way Analysis of Variance (ANOVA) with four replications, which was performed using the program of SAS software. The probabilities of significance were

Table 1. Means comparison* of chemical composition based on percentages in the EO of *O.vulgare* and *O.majorana* at different growth and developmental stages.

No.	Compound	KI	Beginning of vegetative growth		Full vegetative growth		Beginning of flowering		Mid-bloom		Full flowering	
			O. vulgare	*O. majorana*	*O. vulgare*	*O. majorana*	*O. vulgare*	*O. majorana*	*O. vulgare*	*O. majorana*	*O. vulgare*	*O. majorana*
1	(*2E*)Hexenal	855	1.07^{b}	0.51^{cd}	0.42^{d}	1.42^{a}	0.12^{f}	1.13^{b}	0.59^{c}	0.24^{ef}	0.28^{e}	0.12^{f}
2	Sabinene	975	4.56^{c}	5.87^{a}	4.66^{c}	5.03^{bc}	1.49^{e}	4.70^{c}	2.42^{d}	5.40^{ab}	2.14^{d}	5.34^{ab}
3	(1)Octen-3-ol	979	4.36^{b}	-	5.32^{a}	-	2.07^{d}	-	4.73^{b}	-	3.62^{c}	-
4	Myrcene	991	1.79^{b}	2.01^{ab}	1.66^{b}	1.62^{b}	0.58^{c}	0.76^{c}	0.90^{c}	2.09^{ab}	0.94^{c}	2.47^{a}
5	α-Terpinene	1017	7.32^{abcd}	10.39^{a}	6.13^{bcd}	10.10^{a}	2.64^{e}	8.11^{abc}	4.37^{de}	8.96^{ab}	5.57^{cde}	8.76^{ab}
6	(*ortho*)Cymene	1026	0.76^{b}	0.61^{b}	0.75^{b}	0.62^{b}	0.54^{b}	0.53^{b}	0.78^{b}	1.60^{a}	0.83^{b}	1.75^{a}
7	(*δ-3*)Carene	1030	-	1.19^{b}	-	1.29^{a}	-	1.00^{c}	-	1.26^{a}	-	1.29^{a}
8	β-Phellandrene	1030	1.87^{a}	-	0.54^{c}	-	0.37^{d}	-	0.64^{c}	-	0.87^{b}	-
9	(*Z*-β)Ocimene	1037	1.13^{d}	3.81^{a}	0.12^{e}	3.15^{b}	0.10^{e}	2.36^{c}	0.20^{e}	2.99^{b}	0.22^{e}	3.12^{b}
10	(*E*-β)Ocimene	1050	0.24^{c}	3.42^{a}	0.20^{c}	3.10^{a}	0.16^{c}	2.34^{b}	0.29^{c}	3.41^{a}	0.31^{c}	3.53^{a}
11	γ-Terpinene	1060	3.23^{f}	16.22^{a}	3.12^{f}	15.61^{ab}	1.59^{g}	13.94^{d}	4.32^{e}	14.40^{cd}	4.19^{e}	15.26^{bc}
12	(*cis*)Sabinene hydrate	1070	-	0.72^{b}	-	0.69^{b}	-	0.57^{c}	-	0.82^{a}	0.09^{d}	0.83^{a}
13	Terpinolene	1089	2.47^{c}	3.72^{a}	2.40^{c}	3.50^{ab}	0.31^{e}	2.59^{c}	0.45^{de}	3.09^{b}	0.79^{d}	3.41^{ab}
14	Linalool	1097	1.69^{c}	4.06^{a}	0.89^{de}	3.67^{ab}	0.82^{e}	3.33^{b}	1.09^{de}	3.81^{ab}	1.46^{cd}	4.08^{a}
15	(*trans*)Sabinene hydrate	1098	-	2.73^{bc}	-	2.37^{c}	-	2.36^{c}	-	3.00^{ab}	-	3.40^{a}
16	(*cis-para*)Menth-2-en-1-ol	1122	-	1.95^{b}	-	1.92^{b}	-	1.89^{b}	-	2.63^{a}	-	2.82^{a}
17	(*trans-para*)Menth-2-en-1-ol	1141	-	1.36^{b}	-	1.36^{b}	-	1.25^{b}	-	2.14^{a}	0.34^{c}	2.09^{a}
18	Terpinene-4-ol	1177	6.27^{c}	25.65^{a}	0.57^{c}	23.09^{a}	5.15^{c}	23.11^{a}	7.05^{c}	22.15^{a}	12.99^{b}	21.81^{a}
19	α-Terpineol	1189	0.47^{e}	5.13^{b}	0.42^{e}	4.53^{c}	0.37^{e}	4.65^{bc}	0.60^{e}	4.99^{bc}	1.56^{d}	6.39^{a}
20	β-Bourbonene	1388	0.91^{c}	-	1.33^{b}	-	1.95^{a}	-	1.47^{b}	-	1.35^{b}	-
21	(*trans*)Caryophyllene	1419	11.51^{d}	1.95^{f}	11.71^{d}	1.98^{f}	21.46^{a}	7.02^{e}	18.45^{b}	3.69^{f}	15.09^{c}	1.05^{f}
22	β-Gurjunene	1434	0.38^{c}	-	0.39^{c}	-	0.57^{a}	-	0.54^{ab}	-	0.51^{b}	-
23	Aromadendren	1441	-	-	-	-	0.42^{a}	-	0.40^{a}	-	0.27^{b}	-
24	α-Humulene	1455	2.72^{bc}	0.09^{d}	3.04^{b}	0.09^{d}	3.62^{a}	0.19^{d}	2.74^{bc}	0.15^{d}	2.54^{c}	0.12^{d}
25	Germacrene D	1485	16.31^{bc}	-	17.36^{b}	-	18.99^{a}	-	17.26^{b}	-	15.73^{c}	-
26	epi-Bicyclogermacrene	1499	2.22^{a}	-	2.10^{b}	-	-	-	-	-	-	-
27	Bicyclogermacrene	1500	3.10^{bc}	2.10^{d}	3.74^{ab}	2.26^{d}	4.00^{a}	4.06^{a}	3.78^{ab}	2.67^{cd}	3.75^{ab}	1.19^{e}
28	(*E,E*-α) Farnesene	1506	6.53^{b}	-	6.13^{b}	-	9.12^{a}	-	6.47^{b}	-	2.48^{c}	-
29	δ-Cadinene	1523	2.09^{b}	-	2.08^{b}	-	2.69^{a}	-	2.67^{a}	-	2.20^{b}	-
30	Spathulenol	1578	1.64^{ab}	0.16^{e}	1.94^{a}	1.21^{bc}	1.93^{a}	1.72^{a}	1.02^{cd}	0.14^{e}	0.56^{de}	0.11^{e}
31	Caryophyllene oxide	1583	1.45^{ab}	-	1.50^{ab}	-	1.60^{a}	1.81^{a}	1.16^{b}	0.09^{d}	0.66^{c}	0.08^{d}
32	α-Cadinol	1654	2.06^{b}	-	2.84^{a}	-	2.37^{b}	-	1.42^{c}	-	1.25^{c}	-
33	EO	-	0.14^{f}	1.25^{b}	0.14^{f}	1.23^{b}	0.13^{f}	1.09^{c}	0.34^{e}	1.38^{a}	0.67^{d}	1.50^{a}

*Means in each row followed by the same letter (a-g) are not significantly different according to Duncan's multiple range test at the 5% level of probability. The obtained values were expressed as mean from three replications.

used for testing the significance among the species during five growth stages. Then, the differences between means were compared by Duncan's multiple range test at 95% confidence interval.

Results

Seasonal variations of the isolated chemical composition in EO from *O. vulgare* and *O. majorana* were analyzed by GC and GC/MS, and the results were shown in Table 1. Analysis of variance for the essential oil content and composition demonstrated that there were statistically significant differences between the both species ($p \leq 0.01$) and different stages of harvest ($p \leq 0.01$, and $p \leq 0.05$). The results showed that 88 different compounds were detected in total of both species. In respect of comparative evaluation in the essential oil composition, only 10 and 49 compounds were in the *O. majorana* and *O. vulgare*, respectively, while 29 compounds were common in both species (Table 1).

Overall, 78 active components were known in EO of the

Table 2. Means comparison* of compound groups based on percentages in the EO of *O.vulgare* and *O.majorana* at different growth and development stages.

No.	Compound	Beginning of vegetative growth		Full vegetative growth		Beginning of flowering		Mid-bloom		Full flowering	
		O. vulgare	*O. majorana*	*O. vulgare*	*O. majorana*	*O. vulgare*	*O. majorana*	*O. vulgare*	*O. majorana*	*O. vulgare*	*O. majorana*
1	Monoterpene hydrocarbons	25.39^{c}	54.43^{a}	21.44^{cd}	50.53^{a}	9.33^{f}	42.53^{b}	16.28^{e}	52.33^{a}	18.51^{de}	55.08^{a}
2	Oxygenated monoterpenes	8.63^{d}	37.39^{ab}	8.03^{d}	34.72^{b}	6.44^{d}	34.10^{b}	8.74^{d}	35.62^{ab}	20.03^{c}	38.56^{a}
3	Sesquiterpene hydrocarbons	48.16^{c}	4.96^{e}	51.03^{c}	5.25^{e}	69.18^{a}	14.60^{d}	60.06^{b}	7.64^{e}	50.46^{c}	3.54^{e}
4	Oxygenated sesquiterpenes	7.99^{b}	0.16^{e}	9.53^{a}	1.21^{e}	9.94^{a}	3.53^{d}	5.50^{c}	0.23^{e}	3.07^{d}	0.19^{e}
5	Others	7.07^{a}	1.91^{d}	7.32^{a}	5.85^{b}	3.26^{c}	3.30^{c}	6.95^{a}	1.53^{d}	5.52^{b}	1.10^{d}

*Means in each row followed by the same letter (a-g) are not significantly different according to Duncan's multiple range test at the 5% level of probability. The obtained values were expressed as mean from three replications.

O. vulgare. Many components were separately identified in the beginning of vegetative growth (52), full vegetative growth (50), beginning flowering (55), mid-bloom (50), and full flowering stage (69) which these components were 97.24, 97.36, 98.16, 97.53, and 97.59% of EO in the O. *vulgare*, respectively. The major components in the EO were germacrene D and *(trans)*caryophyllene in the beginning of vegetative growth, full vegetative growth, and full flowering stage and vice versa, (trans)caryophyllene and germacrene D in the beginning flowering, and mid-bloom stage (Table 1). Overall, 39 active components were recognized in EO of the *O. majorana*. Several components were separately indicated in the beginning of vegetative growth (37), full vegetative growth (37), beginning flowering (37), mid-bloom (38), and full flowering stage (39) that these components were 98.84, 97.85, 89.09, 97.36, and 98.46% of EO in the *O. majorana*, respectively. The main components in the EO were terpinene-4-ol and γ-terpinene in all growth stages, respectively (Table 1).

Analysis of variance on the categories of the compounds revealed that there were statistically significant ($p \leq 0.01$) differences between the both species in different stages of harvest. According to the comparison of the means, the highest content of compounds were obtained for sesquiterpene hydrocarbons and oxygenated sesquiterpenes in the beginning of flowering stage, and also oxygenated monoterpenes in the full flowering stage in both species. The maximum amount of monoterpene hydrocarbons in EO of *O. vulgare* and *O. majorana* were observed in the beginning of vegetative growth and full flowering stage, respectively (Table 2). Maximum amount of total monoterpenes with values of 38.54% (*O. vulgare*) and 93.64% (*O. majorana*) and total sesquiterpenes with values of 79.12% (*O. vulgare*) and 18.13% (*O. majorana*) was respectively related to the full flowering and beginning flowering stage. Ratio of total monoterpenes/total sesquiterpenes (0.72 and 25.10) was also associated to the full flowering stage in both species (Table 2). With a general account, sesquiterpene compounds had the maximum amounts in EO of *O. vulgare* in five stages of growth especially in the beginning of flowering stage. In contrast, monoterpene compounds had maximum value in EO of *O. majorana* (Table 2).

Discussion

Comparison of the obtained EO from aerial parts of *O. vulgare* and *O. majorana* in different stages showed that the maximum value of EO (0.67%, and 1.5%, respectively) reached in the final harvesting time. In this regard, our results are consistent with the findings of Baranauskien et al. (2013). They studied the effect of harvesting time on yield and EO components in two subspecies of the *O. vulgare* growing in Lithuania which the highest value of EO (0.56%) obtained in the full flowering stage. Our analysis showed that the maximum EO being reached during full flowering stage in both species. Furthermore, Selami et al. (2009) studied the EO content of *O. majorana* growing in the Tunisia and determined the maximum of EO being during full flowering stage. Many studies were done on aromatic plants and all of them indicate that the maximum content of EO being during full flowering stage and end of flowering stage such as Ozguven and Tansi (1998) on *Thymus vulgaris*, Mallavarapu et al. (1999) on *Artemisia pallens*, Sefidkon et al. (2007) on *Satureja rechingeri*, Kizil et al. (2008) on *Origanum onites*, and Verdian (2008) on *Laurus nobilis*. Decrease in the EO in vegetative growth stage can be caused by low speed biosynthesis of volatile compounds during this stage which may be due to the relative inactivity of enzymes required for the synthesis of certain compounds.

Naghdi Badi et al. (2004), and Oliveira et al. (2005) reported that yields of EO during the development stages increasing and reaches its maximum in the flowering stage.

Comparison of the chemical composition in the EO of *O. vulgare* and *O. majorana* in five different development stages showed significant differences in major components. Galoburda et al. (2008) studied the compounds in EO of *O. vulgare* in development stages and determined that sabinene, (*trans*)caryophyllene, germacrene D, and (*Z*-β)-ocimene been the major constituents of the EO, while in our study germacrene D, (*trans*)caryophyllene, terpinene-4-ol, α-terpinene, and γ-terpinene were major constituents. Content and compounds of the EO varied with phenological changes that matched with other studies on Lamiaceae family species such as *Salvia officinalis*, *Salvia fruicosa*, *O. vulgare*, and species from other aromatic families such as *Pelargonium graveolens* (Putievsky et al. 1986; Ravid and Dudai 1988; Ravid and Putievsky 1984). Raina and Negi (2012) studied on the two EO components of *O. vulgare* and *O. majorana* in India and determined that thymol, carvacrol, γ-terpinene and *p*-cymene were the major components of the *O. vulgare*, which thymol and carvacrol made up 40% of EO while these compounds were quietly absent in the *O. majorana*. But in present study, germacrene D, (*trans*)caryophyllene, terpinene-4-ol, and α-terpinene in the *O. vulgare* and terpinene-4-ol, γ-terpinene, α-terpinene, and α-terpineol in the *O.majorana* were the main constituents. Comparing the results with previous research (Rohloff et al. 2005; Mirjalili et al. 2006; Telci and His 2008) suggesting that physiological processes and harvesting time significantly affected the EO components. Komaitis (1992) determined that terpinene-4-ol was the main compound of EO in the Greece (37%) and then was *p*-cymene (12.05%). Trevino and Johnson (2000) also achieved a similar result about the *O. majorana*. Vera and Chane (1999) studied the EO of *O. majorana* grown in the Reunion Island and determined that the main components included terpinene-4-ol (38.4%), (*cis*)sabinene hydrate (15.0%), *p*-cymene (7.0%), and γ-terpinene (6.9%). The analyzed EO of *O. majorana* grown in the Argentina by Branchio et al. (2008) had general similarity to the present EO, terpinene-4-ol (55.09%), (*trans*)sabinene hydrate (13.2%), α-terpineol (9.09%), and (*cis*)sabinene hydrate (8.37%). Diversity in the EO compounds of *O. majorana* at different regions can be caused by interaction of genetic and environmental factors. Ricci et al. (2005) determined that the EO compounds of aromatic plants in particular were influenced by environmental factors. Also, Sangwan et al. (2001); Kim and Lee (2004) believed that the EO compounds were influenced by many factors, including genetic, development stages, extraction method and assay conditions. Baranski et al. (2005) determined that the subspecies of *O. vulgare* grown in the Lithuania were related to chemotype sabinian, β-ocimene, β-caryophyllene and germacrene D. This opinion was resembled with present results and also with previous studies (Radusien et al. 2005; Mockute et al. 2001).

Comparison of the classified compounds in EO of *O. vulgare* and *O. majorana* in five different development stages showed significant differences in the compounds groups. In the beginning of flowering, the level of monoterpenes hydrocarbons drastically reduced and sesquiterpenes hydrocarbons had the maximum amount of EO and then in flowering stage, monoterpenes hydrocarbons were increased again. Researchers like Sarir et al. (1982); Jolivet et al. (1971); Baser et al. (1993); Sellami et al. (2009) stated that *O. majorana* species studied in the different regions of world. There were two main chemotype: a chemotype mostly monoterpenes alcohol and other included complex of phenols. In the first category terpinene-4-ol with other monoterpenes alcohol, such as (*cis* and *trans*)sabinene hydrate formed the major component of EO and the second group, the main component consisted mainly of thymol and/or carvacrol. However, our results showed that over 90% of EO components in the *O. majorana* were monoterpenes and unlike to previous research reports. The amounts of phenolic compounds were very low that matched with studies on the *O. majorana* which cultivated in the Finland (Vera and Chane 1999). Precursors of the phenolic compounds such as *p*-cymene, and γ-terpinene in the *O. vulgare* were the greater than *O. majorana*, while sabinian, (*cis and trans*)sabinene hydrate, and α-terpineol were the higher in *O. majorana*. Also, amount of the oxygenated monoterpenes were much more in the *O. majorana*. It seems the high content of terpinene-4-ol due to rearrangements occurs during the distillation process (Sangwan et al. 2001; Kim and Lee 2004).

Conclusions

Since, all samples of both species in this study were cultured in the same climatic conditions. Therefore, the changes of content and composition in the EO were relevant to the genetic differences between species in dealing with seasonal changes. By focusing on the EO and their compounds, our results showed that maximum amount of EO of *O. vulgare* and *O. majorana* were obtained in the full flowering stage. In the EO of *O. vulgare*, germacrene D was the major component that its maximum amount was detected in the beginning flowering stage. In the *O. majorana*, terpinene-4-ol was the major component of EO that its maximum amount was identified in the beginning of the vegetative growth. Sesquiterpene compounds had the maximum amounts in EO of *O. vulgare* in five stages of growth, especially in the beginning of flowering stage. In contrast, monoterpene compounds had the maximum value in EO of *O. majorana* in the full flowering stage. *O. majorana* is good source of EO, especially in full bloom.

The EO of *O. vulgare* is also important as it contains important bioactive compounds such germacrene D, and (*trans*) caryophyllene that would be harvest in a good time to achieve maximum concentration of these compounds in the EO and being exploited in pharmaceutical and food industries.

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ARTICLE

Morphological identification of haematopoietic cells in pronephros of common carp (*Cyprinus carpio* Linnaeus, 1758)

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ABSTRACT Haemopoietic tissue of the common carp pronephros (*Cyprinus carpio* Linnaeus, 1758) was studied in regard of morphometric analysis of the erythropoietic, leukopoietic and thrombopoietic cell populations. The aim of this study was to perform evaluation of immature precursor cells in head kidney of common carp (sampled from Bardaca lake) because there is no well known data regarding this issue. Microscopic identification of haematopoietic cells included measurement of cell and nuclear size, nuclear-cytoplasmic ratio, determination of the cell and nuclear shape, cytosol coloration and presence of specific granules in the cytosol. The frequency of immature cells and their cell area was also analyzed. Erythroblasts were the most abundant among all observed haemopoietic cell lineages and were the most variable in the size. The largest area was characteristic of monocyte precursors and no significant differences were observed regarding the cell area between prothrombocytes and lymphoblasts, which makes difficult in cell characterization. High number of emerged cells in short time also makes difficult to identify particular stages of maturation in some bloodlines. Rapid maturation of granuloid cells observed within the haemopoietic tissue indicates their functional significance in adaptation to the changeable microenvironment. **Acta Biol Szeged 60(2):113-118 (2016)**

KEY WORDS

common carp
haematopoiesis
pronephros
cell-lineage
cell area

Introduction

The common crap, *Cyprinus carpio* Linnaeus, 1758 is a freshwater cyprinid fish of eutrophic waters in Europe. Although tolerant to most environmental changes, especially low oxigen levels, common carp prefers slow waters and vegetative sediments as their habitat (Rey et al. 2016). In such conditions they are exposed to attacks of pathogens such as bacteria and parasites, they ought to have well-developed defence mechanisms. In many fish species blood cells play a major role in providing immunity and therefore the process of haematopoiesis is very crucial (El-Saydeh et al. 2010; Prajeena et al. 2014). Similarly to most teleost fish (Homechaudhuri and Jah 2001; Huang and Zon 2008; Chen and Zon 2009; Paik and Zon 2015; Alijagic and Suljevic 2016b), pronephros has major role in carp haematopoiesis, in spite of other organs which may also show hematopoietic activity such as mesonephros (Stosik and Deptula 1993), thymus, spleen (Patel et al. 2002) and serosa of the middle part of the intestine (Stosik and Deptula 1993). However, pronephros did not show only hematopoietic activity, it is also the reservoir of cells, a lymphoid and endocrine organ (Wendelaar Bonga 1997; Weyts et al. 1999).

Haemopoietic stem cells are placed along the urinary tubules in kidney marrow. Directed stem cells derive from hemogenic endothelium and differentiate in several haemopoietic lineages (Willett et al. 1999). The pronephric kidney functions as main erythropoietic, lymphomyeloid and thrombopoietic organ in common carp (Kobayashi et al. 2007; Kondera 2011). In juvenile common carp pronephros the blood cell production is consisted of the following cell lines: unidentified blast cells, erythroid, granuloid, lymphoid, monocytoid and thrombocytoid cells (Kondera 2011). Haematopoiesis in common carp consists of 22 blood cell types, which can be easily identified and counted. They can be observed in different stages of maturation and the differences between them are based on various nuclei size, ratio between nucleus and cytoplasm or chromatine condensation (Alijagic and Suljevic 2016b). A combination of quantitative and morphological methods is needed thus it can be done by flow cytometry and microscopically. The combined use of flow-cytometry and electron microscopy makes it possible

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to characterize different cell types and to monitor changes in blood cell populations (El-Saydah et al. 2010).

All haematopoietic cell types in fish are similar to those in mammals, even though mammalian myeloid cell-line proliferation and differentiation predominantly occurs in bone marrow (Kobayashi et al. 2007; Chen and Zon 2009; Ivanovski et al. 2009) unlike haematopoiesis in fish that occurs in several different organs. Basic mechanisms found in vertebrates are also active and functional in channel catfish (Fijan 2002a; Fijan 2002b).

The analogous expression of different markers in zebra fish and other vertebrate models suggests that the molecular mechanisms that regulates haematopoiesis are highly conserved. Consequently maturation of hemangioblast occurs accompanied with the interaction of factors like Scl and Lmo2 (Patterson et al. 2007). Definitive haematopoiesis include Fli1-A, Hhex and Tbx16 transcription factors. Transcription factor with ETS-domain (Fil-A) is implicated in proliferation or differentiation of haematopoietic precursors (Brown et al. 2000). Once the haematopoietic precursors have been specified, additional haematopoietic transcription factors such as Gata1, Pu.1, and Ikaros direct the lineage-specific differentiation of these progenitors into erythroid, myeloid, and lymphoid cell types, respectively (Huang and Zon 2008).

Several researches showed quantitative data on the proportions between blood cell lineages in haematopoietic organs of teleosts (Peters and Schwarzer 1985; Wlasow and Dabrowska 1989; Fijan 2002a; Fijan 2002b; Kondera 2011; Alijagic and Suljevic 2016a; Alijagic and Suljevic 2016b). The aim of the presented study was to perform quantitative and qualitative evaluations of developing blood cells in the pronephros of common carp, *Cyprinus carpio* Linnaeus, 1758.

Materials and Methods

Site

Fish sampling was done on Bardaca lake, which is located in nothern part of Bosnia and Herzegovina. Hydrological status of lake mainly depends upon three rivers: Matura, Stublaja and Brzaja. Lake is also part of Barda a reservoir (45°06 33 N 17°26 07 E / 45.10917°N 17.43528°E), which consists of eleven lakes and it is used as a fishpond.

Sampling and experimental design

The sample consisted of 20 specimens (10 females and 10 males) with an average weight of 18.05 ± 4.105 g (WBW digital scale) and lenght of 12.22 ± 1.299 cm. Fishnets (Attwod Fold-N-Stow) were used in sampling. After sampling, fish were transported from Bardaca lake to the Laboratory of Physiology (Faculty of Science, Sarajevo, Bosnia and Herzegovina) in containers supplied with pure oxigen (water aerators, CHAMPIONCX- 0098) and allowed to acclimate to the laboratory tank for 15 days. Water temperature was 20° C, pH 7.1, oxygen saturation level was 80%. Water monitoring included daily water changes, measurement of oxygen (Winkler method) and ammonia concentration (Nessler method). The fish were fed twice a day formulated Eco FeedEx C 48/10 (Eco Feed Ltd, Serbia).

Kidney biopsy and haematological methods

Fish were euthanized with 0.2% tricaine (Penta Chemicals) prior kidney biopsy and placed in ice-cold water for 4 to 5 minutes. After euthanisation they were dissected using sharp scissors, internal organs were removed from abdominal cavity and the head kidney was collected (approximately 0.5 cm^3) for the preparation, using biopsy tweezers. The surface of isolated fresh organs was smeared gently (slight zig-zag movements with pin) on fatfree slides. After being dried for 24 h, tissue smears were treated by the Leders method (Penta Chemicals) to prove the presence of peroxidase activity in granuloblasts. Smears were stained using May-Grünwald and Giemsa solutions (Pappenhein staining method).

Microscopic analysis

Identification were performed using a light microscope Olympus BX41 and all measurments (number and area) of haemopoietic cells were performed using the Olympus DP12 camera, all photos were imported into Olympus DP Software. The results were presented as percentages of the total number of blood cells. Area of cells occupied by the nucleus was estimated visually. Morphological characterization of haematopoietic cell-lines included determination of cell and nuclear shape, measurement of cell and nuclear size, level of chromatine condensation, nuclear-cytoplasmic ratio, cytosol coloration and presence of specific granules in cytosol. Measurements for all individuals were determined as the mean value of each cell type in a sample of 300 cells.

Statistical analysis

Analysis were performed using SPSS (Version 20.0, SPSS, Inc., Chicago, IL, USA), and data are presented as means ± 1 SD accompanied by range and coefficient of variation (%).

Results

In this research 20 juvenile healthy specimens of common carp *Cyprinus carpio* (10 males and 10 females) were exam-

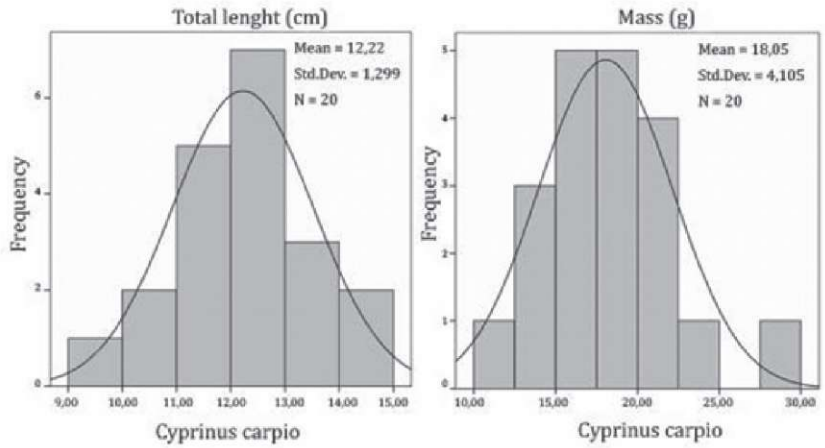

Figure 1. Total lenght and body mass of common carp (*Cyprinus carpio* Linnaeus, 1758).

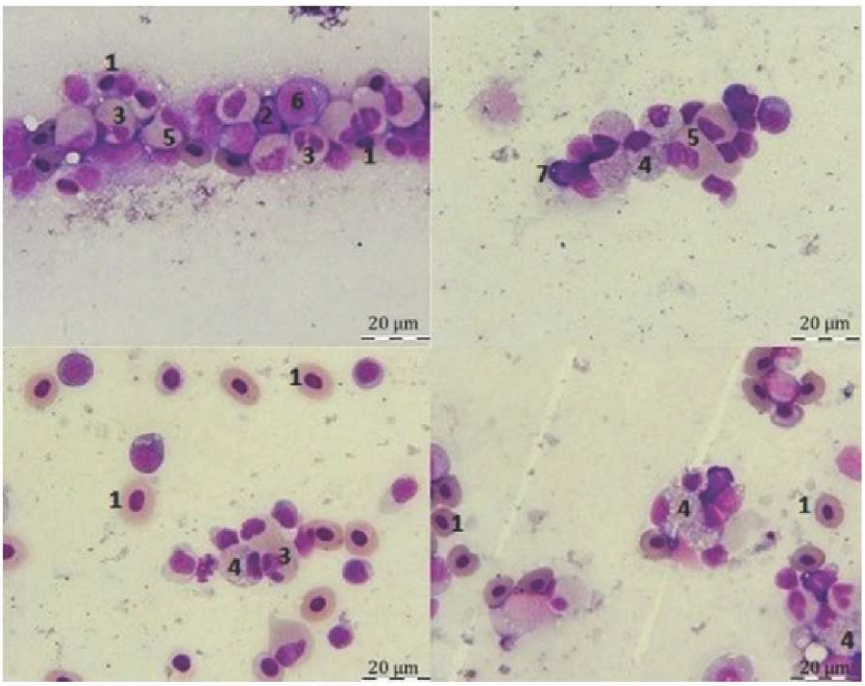

Figure 2. Immature cells in pronephros of common carp: erythroblast (1), lymphoblast (2), neutrophilic metagranulocyte (3), heterophilic metagranulocyte (4), granuloblast (5),monocyte precursore (6), prothrombocyte (7).

ined. The average total length of the specimens was 12.22 ± 1.29 cm and in most of them (seven) amounted value was 12 cm, while the average body mass was 18.05 ± 4.10 g, in which ten specimens had weight in range of 15 to 20 g (Fig. 1).

We characterized the immature elements of the haematopoietic hierarchy formed by haematopoietic stem cells: erythroblast, lymphoblast, neutrophilic metagranulocyte, heterophilic metagranulocyte, granuloblast, monocyte precursore and prothrombocyte.

Hematopoietic cells and their characteristics identified by using light microscopy and cytochemical POX method are presented in Figure 2. and Table 1.

Measures of central tendency, range and coefficient of variation (CV) for all analyzed immature cells were presented in Table 2. The most prevalent were erythroblasts with highest SD. The following were heterophilic metagranulocytes, lym-

Table 1. Appearance (shape and staining property) of the haematopoietic cells in the pronephros of common carp (Pappenhein staining method).

Cell type	Shape		Staining property	
	Cell	Nucleus	Cytoplasm	Nucleus
Erythroblasts	oval	oval	red	deep blue
Lymphoblasts	round	round	blue	deep blue
Neutrophilic metagranulocytes	irregular	irregular or band	light red	violet
Heterophilic metagranulocytes	spherical	eccentric; round, elongated, irregular	light violet with granules	violet
Granuloblasts	irregular	irregular or band	light violet	violet
Monocyte precursores	spherical	spherical or elongated	light blue	violet
Prothrombocytes	round	round	blue	deep blue

Table 2. Frequency (%) and values (number and area) of determined haematopoietic cells in pronephros of common carp.

Cell type	Number				Area (μm²)		
	%	Mean±SD	Range	CV	Mean±SD	Range	CV
Erythroblasts	35.26	105.80±19.08	72-150	18.03	82.32±18.22	57.33-125-58	23.14
Lymphoblasts	11.40	34.20±7.54	21-51	22.04	77.11±11.94	56.57-96.96	15.48
Neutrophilic metagranulocytes	9.91	29.75±7.90	12-42	26.55	116.10±15.60	78.68-134.35	13.44
Heterophilic metagranulocytes	22.28	66.85±9.79	54-99	14.64	120.29±11.44	109.12-154.8	9.51
Granuloblasts	7.21	21.65±6.23	12-33	28.80	110.40±8.08	90.97-123.25	7.23
Monocyte precursores	6.00	18.00±4.57	9-24	25.63	155.10±8.57	143.30-177.64	5.52
Prothrombocytes	7.91	23.75±6.05	15-36	25.48	44.63±9.13	24.73-55.06	20.46

phoblasts, neutrophilic metagranulocytes and granuloblasts, respectively. Prothrombocytes and monocyte precursors were less presented. Table 2. presents the area (μm²) of various hematopoietic cells including the mean, range and CV.

Granuloblast characterized the smallest, whereas erythroblasts had the highest SD, which indicates that the erythroblasts were the most variable in their size. According to the range and CV, the largest variations in size were obtained in erythroblasts and prothrombocytes. Otherwise, the smallest variation in area of hematopoietic precursors were characteristic of monocyte precursors and granuloblasts. Therefore identification of immature cells was based on differences in morphological characteristics (Fig. 1).

Discussion

Maturation of haematopoietic cells in fish differs compared to haematopoiesis in other organisms. Head kidney is a main organ forming the blood elements in fish and its role in the blood elements formation differs among teleost fish (Willett et al. 1999; Rombout et al. 2005). It can be organ-forming erythroid lineages only, or it produces all types of blood cells (Meseguer et al. 1990; Willett et al. 1999; Esteban et al. 2000; Stephens et al. 2004; Kondera 2011). Large cell production in short time make difficult to identify immature cells, especially the number of particular stages of maturation in some lineages (Kondera 2011). In the present study, developed stages were not analyzed but only immature precursors.

Erythroblasts were the most abundant among all observed haemopoietic cell lineages in this study (13.14 ± 1.00%). The percentage of erythroid lineage cells was similar to the results obtained by (Wlasow and Dabrowska 1989) for common carp and (Peters and Schwarzer 1985) for rainbow trout (near 37% and near 45%, respectively). Results were considerably lower in relation to the results obtained by Kondera (2011) for common carp and Fijan (2002a) for channel catfish (13.14 ± 1.00% and 13.0 ± 5.1%, respectively). Lymphoblasts were highly present among all observed haemopoietic cell lineages, after heterophilic metagranulocytes. The percentage of lymphoid cells in the head kidney of the same fish species often differs. In the present study, the frequency of lymphoblasts (11.4%) in common carp was similar to the percentage of lymphoid cells (8.5%) in research observed by Wlasow & Dabrowska (1989), but a smaller amount of lymphoblasts was reported in pronephros for channel catfish and common carp (Fijan 1961; Fijan 2002b; Kondera 2011).

Cells with high selfrenewal potential and high division rate are granuloblasts. The heterophilic metagranulocytes were the most frequent granuloid cell (22.28%). In tench pronephros, pseudoeosinophilic granuloblasts are the most

numerous leukocytes (Alijagic and Suljevic 2016b). Neutrophilic metagranulocytes (9.91%) were more abundant than shown by Fijan (2002b) in channel catfish (4.53 ± 1.53%, neutrophilic progranulocyte and neutrophilic metagranulocyte were counted together) by Wlasow and Dabrowska (1989) and Kondera (2011) in common carp (only neutrophilic progranulocytes were counted 3.40 ± 1.62% and 8.40 ± 0.97%, respectively). The small number of granuloblasts (7.21%) probably was a result of their rapid maturation that have impact on their characterization.

The frequency of monocyte precursors (6%) differs compared to other reports (Fijan 2002a; Kondera 2011). In the head kidney of carp 0.98 ± 0.45% of monocytoid cells were observed and it similarly for channel catfish 0.91 ± 0.82%.

The prothrombocytes were round, with round nucleus which occupies the entire cell, and size of average lymphoblasts. In the present study, number od prothrombocytes found in the head kidney (7.91%) was similar to number of trombocytes in head kidney of channel catfish (Fijan 2002a), but higher compared to results observed in common carp (Kondera 2011).

A small number of scientific papers are published on the topic of size of haematopoietic precursors. Based on the results it can be concluded that the area and thus the size of haematopoietic cells varies a lot. The variability in erythroblasts area was probably because of presence of the different maturation stages, in which ratio between longer and shorter axis of the cell is changeable, as well as nuclear-cytoplasmic ratio. Based on the color of the cytoplasm it is difficult to observe each stage of erythroblast maturation. Cell-nuclear ratio, nuclear shape and size, as the key morphological changes during the maturation of erythroblasts should be included in cell characterisation, both for erythroide and other haematopoietic cell lineages.

No significant differences were observed regarding the cell area between prothrombocytes and lymphoblasts (they also were the smallest identified cells) which make difficult in distinguishing these cells (Kondera 2011). Therefore, prothrombocytes could be classified as leukocyte precursors.

The largest area was characteristic of monocyte precursors (155.10 ± 8.57 μm^2). The myelocyte and metamyelocyte as observed by Diago et al. (1998), and the promyelocyte and myelocyte reported by Wlasow and Dabrowska (1989) are similar to heterophilic metagranulocytes and neutrophilic metagranulocytes as the largest cells in myeloid cell lineage (120.29 ± 11.44 μm^2 and 116.10 ± 15.60 μm^2, respectively). Granuloblasts as myeloid precursors had smaller area; also CV showed no significant individual variations.

In fish, analysis of cell associations in the haemopoietic tissues is of great importance in the study of haemopoiesis and the formation of haemopoietic microenvironments (Gangopadhyay and Homechaudhuri 2011). Romano et al. (2002) studied the histology of the head kidney in two Antarctic fish species and observed a difference in the shape of erythrocytes, increased number of granular and lymphatic cells, and this is considered an adaptation to the function of the pronephros at low temperatures.

The complete blood count is the good indicator of environmental or stress impacts in common carp (Kondera and Witeska 2013). The presence of mature cells in pronephros occurs due to the lack of oxygen in water and it is related with good adaptive mechanisms. This phenomenon would occur much slower if the morphological differentiation followed separate stages of erythropoiesis (Alijagic and Suljevic 2016b). However, little data are available on the effects of environmental factors on haematopoiesis in common carp, which is sensitive to various impacts due to its high production rate of blood cells.

In conclusion, the cellular composition of hematopoietic tissue in same and evolutionary close fish species is similar, but there are quantitative differences. Morphological parameters like cell and nuclear size, nuclear-cytoplasmic ratio and shape, cytosol coloration and presence of specific granules in cytosol are good indicators for microscopic identification of immature cells. However, unification of nomenclature and the cell differentiation criteria is essential in the study of fish haematopoiesis.

The presence of various immature cells in carp is a very unstable parameter that depends on many factors, including environmental factors like physiscal and chemical water parameters, water contamination, parasites as biological stressors and diseases. Frequency of various haematopoietic cells that vary even in the same fish species and rapid cell production in specific environmental conditions reflects the adaptation of organisms to variable environmental conditions. Surely, further researches are needed as an additional confirmation of these assumptions.

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ARTICLE

A study of some biocontrol mechanisms of *Beauveria bassiana* against *Rhizoctonia* disease on tomato

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ABSTRACT Tomato damping-off, caused by *Rhizoctonia solani*, is one of the most common diseases worldwide. The use of biological control agents to protect plants against pathogens seems to be an appropriate solution. *Beauveria bassiana* as an endophytic fungus can colonize a wide range of plants in a systemic manner and enhance plant resistance. In this study, the ability of three isolates of *B. bassiana* to control damping-off of tomato seedlings was examined. The effect of *B. bassiana* on the induced systemic resistance enzymes such as peroxidases (POX) and phenylalanine ammonia-lyase (PAL) and also the phenolic compounds content in plant resistance was measured. Our results showed that *B. bassiana* isolates especially TS12 and TS7 resulted in an increase in the activity of POX and PAL enzymes as well as enhanced the contents of phenolic compounds in treated plants. The results of greenhouse studies showed that each of the three isolates were able to successfully control tomato damping-off. However, this ablity was probably due to the stimulation of plant growth by KJ24, but TS12 and TS7 isolates acted through the stimulation of "induced systemic resistance" in plants.
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KEY WORDS

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biocontrol
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Introduction

Tomato is one of the most consumed and important vegetables and field crops in the world (Blancard et al. 2012). Apart from being daily consumed, this plant is an important model organism, the complete genome sequence of which has also been determined (The Tomato Genome Consortium 2012). Damping-off and root rot of tomato caused by *Rhizoctonia solani* is one of the most destructive diseases in some of the tomato production areas worldwide. It is also capable of infecting cultivars of tomato in the greenhouse (Montealegre et al. 2010). The disease causes an average decrease of 20% in the annual yield worldwide (Muriungi et al. 2014). Because of the broad host range and high rates of overwintering sclerotia, the control of *R. solani* is very difficult (Grosch et al. 2006). Considering the damages of chemical pesticides and problems associated with other means of control, it seems that biocontrol could be the perfect solution for protecting plants against this pathogen (Ewekeye et al. 2013). Different types of microorganisms as biocontrol agents have been identified, and, among them, the exploitation of biocontrol fungi is developing (Butt et al. 2001). Different bicontrol mechanisms by biocontrol fungi against plant pathogens have been identified. The biocontrol fungi are directly involved in some of these mechanisms, such as competition, antibiosis, and parasitism. Other mechanisms, including induced systemic resistance (ISR), increased growth response, and endophytic colonization in the plant have an indirect role, and, through the activation of plant defense responses and increased expression of genes related to defense they cause induction of plant resistance and disease reduction (Ownley et al. 2010). Induced systemic resistance is activated by mycorrhizae, as well as some biocontrol (Ownley et al. 2010) and endophytic fungi (Gasoni and Gurfinkel 2009). Colonization of plants by endophytic fungi can be local or systemic (Saikkonen et al. 1998). Systemic colonization can be caused by entomopathogenic fungi and act against plant disease (Ownley et al. 2010). Localized colonization by endophytes could also increase production of phenolic compounds and other defence metabolites in the plants (Gasoni and Gurfinkel 2009). The endophytic activity of *Beauveria bassiana* isolates has been reported from several plant species under natural conditions, as well as from plants including tomato, cotton, green beans, soybeans, corn, datura and potatoes inoculated using various methods (Ownley et al. 2008, 2010). The antagonistic abilities of different isolates of *B. bassiana* were investigated against certain plant pathogens including *Fusarium oxysporum*, *Ar-*

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millaria mellea, Rosellinia necatrix, R. solani, and *Pythium myriotylum* (Griffin 2007). Application of *B. bassiana* isolate 11-98 on tomato seed resulted in the endophytic and epiphytic colonization of seedlings and protection against damping-off (Ownley et al. 2008). Similarly, in cotton (*Gossypium hirsutum* L.) seed, the application of the mentioned isolate reduced *R. solani* damping-off in seedlings via the activation of induced systemic resistance (Griffin 2007). Several studies have characterized *B. bassiana* as a fungus with the potential to control plant diseases, but this is still being investigated by researchers.

Despite the fact that the ability of *B. bassiana* in improving the protection of plants against a number of soil-borne plant pathogens has been reported, most researches performed on the biological control effect by *B. bassiana* on plant diseases were limited to *in vitro* studies. For this purpose, greenhouse tests along with *in vitro* studies as well as the comparison of the virulence and the operation mode of different strains of this fungus may provide important information about its biocontrol mechanisms reducing the impact of the disease. This study has been undertaken to explore the total phenol content and the activities of peroxidase and phenylalanine ammonia-lyase enzymes in addition to greenhouse and laboratory evaluations.

Materials and Methods

Preparation of pathogen and antagonistic isolates

Three antagonistic isolates of *Beauveria bassiana* (TS12, KJ24, and TS7) were obtained from the culture collection of the Plant Protection Department, Azarbaijan Shahid Madani University. *Rhizoctonia solani* (AG-4) derived from the Plant Protection Department, University of Tehran.

Growth and sporulation pattern of B. bassiana isolates

As the growth and sporulation rates of different *B. bassiana* isolates is variable, the rate of mycelial growth of *B. bassiana* isolates was studied on two media, Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA), based on the methodology proposed by Mostafa et al. (2010). To measure the conidial production of the isolates, the test was carried out in the light of the methodology proposed by Senthamizhlselvan et al. (2010) in two liquid media [Potato dextrose broth (PDB) and Sabouraud Dextrose Broth (SDB)]. After five days, the spore concentration in each Erlenmeyer flask was measured using a hemocytometer and analyzed.

Chitinase activity assay

According to Chan and Tian (2005), cell wall of *R. solani* was prepared and chitinase enzyme was extracted. Chitinase activity was assayed using the method of Kang et al. (1999) at a wavelength of 585 nm.

Toxin production assay

This test was performed by the method described by Parveen and Begum (2010). The experiment was carried out in triplicate based on completely randomized design with four treatments and four replications.

Antagonistic effect of B. bassiana to prevent tomato damping-off under greenhouse conditions

Seed treatment was carried out according to Griffin (2007). After surface sterilization, seeds of tomato (*cv.* mobil) were coated with antagonistic fungal spore suspension of three concentrations (10^5, 10^7, and 10^9 spores/ml), separately. Inocula of the pathogen were prepared on wheat seeds as described by Tseng et al. (2008) and added to the soil at a rate of 3% wt/wt (based on soil fresh weight). Then, seeds were planted in potting soil infested with pathogen inocula (three seeds into each pot). After planting, the pots were transferred to a greenhouse (24-27 °C, 30% relative humidity, and 16 h photoperiod). This experiment was arranged as a completely randomized design with 11 treatments (infected control, healthy control, and nine treatments comprising the three antagonist isolates TS7, KJ24, and TS12 with three spore concentrations) and 10 replicates (pot). Three weeks after the cultivation of plants, healthy plants were evaluated according to the damping-off and wilting symptoms and the percentage of "damping-off" of plants was calculated according to the following equation: $D= [(N–N0) / N] \times 100$, where, N is the number of healthy seedlings in control and $N0$ the number of healthy seedlings in each treatment.

In the fourth week, growth factors such as dry and fresh weight and height of plants were measured.

Preparation of plant extracts and determination of enzyme activities

Enzyme assays were performed with leaf and stem extracts. Tomato leaves were collected in the third and fourth weeks after cultivation of plants. Initially extracts of plants were prepared according to the modified method of Abo-Elyosr et al. (2007) and stored at -80 °C until the determination of the activities of peroxidase, phenylalanine ammonia-lyase, and total phenolic compounds.

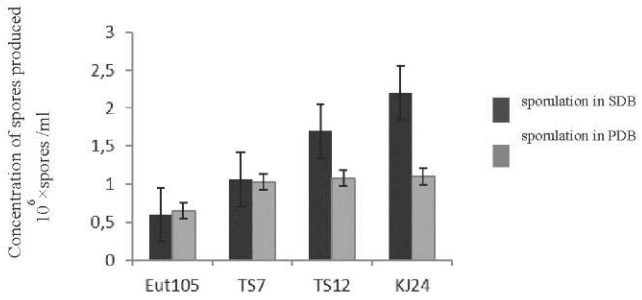

Figure 1. Concentration of spores produced by *B. bassiana* isolates TS12, TS7 and KJ24 in PDB and SDB media at 26±1 °C after 10 days of incubation. The concentration of spores was measured using a hemocytometer. Vertical bars represent ± S.E. of means (n = 3).

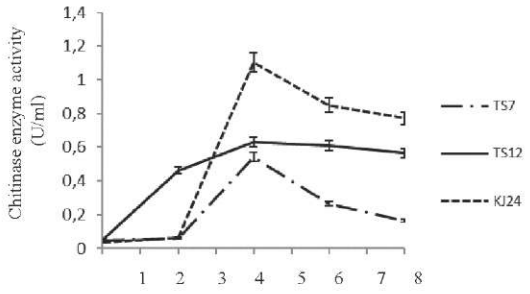

Figure 2. Chitinase enzyme activities of *B. bassiana* isolates TS12, TS7 and KJ24 during eight days of growth at 26±1 °C in Lilly-Barnett (Minimal Salt) medium containing *R. solani* cell wall (2 mg/l) as sole carbon source. Vertical bars represent ± S.E. of means (n = 3).

Peroxidase (POX) activity was determined according to the method of Urbanek et al. (1991) with a little modification. The reaction solution contained 0.5 ml of the enzyme extract, 350 µl phosphate buffer (100 mM), 350 µl pyrogallol (10 mM), and 1 ml of H_2O_2 (70 mM), respectively. Absorption at 470 nm was recorded (extinction coefficient 26.6 mM^{-1} cm^{-1}). POX enzyme activities were calculated as the µMol H_2O_2 decomposed in mg protein per minute.

Phenylalanine ammonia-lyase (PAL) was measured according to the modified method of Pascholati et al. (1986) with cinnamic acid as standard. The reaction solution was obtained by combining the 15 µl of the enzyme extract and 1500 µl phenylalanine (2%). Then the absorption at 290 nm was recorded immediately. Phenylalanine ammonia-lyase enzyme activities were expressed as mg per liter (mg/l) cinnamic acid in mg protein per minute.

The content of phenolic compounds was measured as described in Javanmardi et al. (2003) with a little modification, and based on the color change of phenolic extracts by the folin reagent and sodium carbonate. Absorption was recorded at 765 nm. The contents of total phenolic compounds were calculated using a standard curve obtained with different concentrations of gallic acid expressed as the mg gallic acid per g of tissue fresh weight.

Statistical analysis

The data were analyzed by SAS statistical package version 9.2 and the mean comparisons were made following Duncan's Multiple Range Test at P = 0.01 by MSTATC (version 2.10, Inc, Michigan State University) . The entire experiment was performed three times.

Results

Growth pattern in B. bassiana isolates

On the second, fourth, sixth, eighth, and tenth days after inoculation, the radial growth rate of the antagonistic fungal isolates was measured and compared on PDA and SDA media. Differences could be observed between the examined isolates ($p \leq 0.01$). At both SDA and PDA culture medium, minimum and maximum growth rates were observed in isolates of TS7 and KJ24, respectively (data not shown). Mean comparison of growth rates of isolates on both PDA and SDA showed that isolates TS12, TS7, and KJ24, had the highest growth rates, respectively.

The pattern of sporulation of B. bassiana isolates

In order to compare the pattern of sporulation of the examined antagonistic fungal isolates, amount of spores produced by the isolates was measured on the fifth day after inoculation into SDB and PDB cultures. Mean comparison showed that the amount of spores produced by KJ24 and TS12 isolates in SDB was higher than in PDB, but there was no significant difference in the case of the isolate TS7 between the two media (Fig. 1).

Chitinase enzyme activity and toxin production assay

By using the data obtained from 0, 2, 4, 6, and 8 days, chitinase activity curves were plotted for each isolate of antagonists. The results showed that all isolates of *B. bassiana* produced the maximum enzyme activities 96 hours after inoculation. Maximum and minimum levels of chitinase activities were produced by isolates KJ 24 and TS7, respectively (Fig. 2).

In the toxin production assay, a hyaline zone around the colonies of strains TS7 and TS12 strain was observed after 7

Table 1. Mean comparison of percentage damping-off and plant growth parameters of tomato plants treated with *B. bassiana* strains in the greenhouse.

Treatment	Percentage damping-off	Root length (cm)	Shoot length (cm)	Dry weight (g)	Fresh weight (g)
TS7 (10^5 cell/ml)	82.65 a*	4.50 bc	9.27 bcd	0.419 de	0.036 c
TS7 (10^7 cell/ml)	34.74 g	4.33 bc	12.67 abc	0.404 de	0.038 c
TS7 (10^9 cell/ml)	56.51 e	2.50 c	7.83 de	0.258 e	0.033 c
TS12 (10^5 cell/ml)	78.23 b	4.10 bc	11.07 abcd	0.505 cde	0.046 bc
TS12 (10^7 cell/ml)	34.74 g	5.66 b	11.43 abcd	0.638 bcd	0.068 bc
TS12 (10^9 cell/ml)	69.53 c	4.17 bc	9.50 bcd	0.390 de	0.038 c
KJ24 (10^5 cell/ml)	60.83 d	4.17 bc	9.00 cd	0.353 de	0.032 c
KJ24 (10^7 cell/ml)	52.17 f	11.83 a	13.83 ab	0.966 b	0.093 b
KJ24 (10^9 cell/ml)	21.71 h	12.00 a	15.00 a	1.466 a	0.157 a
Non-infected control	0.00 i	5.67 b	7.50 de	0.796 bc	0.090 b
Infected control	82.62 a	4.43 bc	4.50 e	0.379 de	0.039 c

*Means followed by the same letters in a column are not significantly different.

days of growth on methylene blue agar at 26 °C.

Antagonistic effect of B. bassiana to prevent tomato damping-off in greenhouse

Three weeks after the cultivation of plants, with emergence of the first signs of damping-off, damping-off percentage was calculated (Table 1). The results proved that there was a difference in the effect of the studied isolates on damping-off percentage, root length, fresh and dry weight, as well as shoot length. Mean comparison showed that isolate KJ24 (10^9 spores/ml) and isolates TS12 and TS7 (10^7 spores/ml) had the maximum controlling effect, with 21.73 and 34.74% damping-off, respectively. Isolate TS7 (10^5 spores/ml) with 82.65% damping-off had the minimum control effect (Table 1). Plants treated with isolate KJ24 with a concentration of 10^9 spores/ml had the maximum shoot and root lengths, as well as dry and fresh weight indicating that this isolate controlled tomato damping-off at this concentration. Although there was no difference between the treatments on stem length, all treatments increased the shoot length when compared to the infected and non-infected controls (Table 1).

Preparation of plant extracts for enzyme activity measurements

Plants from treatments with less than 70 percent damping-off were used to determine the activities of peroxidase enzyme, phenylalanine ammonia-lyase, and total phenolic compounds.

Peroxidase enzyme activity

After three and four weeks of tomato cultivation, the peroxidase activities were significantly affected by the treatments. The enzyme activities in all treatments were higher than in case of the infected control in the third week (Table 2). Maximum and minimum activities were observed in plants treated with isolates KJ24 at a concentration of 10^9 spores/ml and TS12 at a concentration of 10^7 spores/ml, respectively. In the fourth week, the enzyme activities were higher than the infected and non-infected control in all treatments, but in some treatments they were increased or decreased in comparison to the values at the third week. Additionally, in the fourth week, the maximum and minimum activities were observed in isolates KJ24 at a concentration of 10^7 spores/ml and TS7 at a concentration of 10^7 spores/ml, respectively (Table 2).

Phenylalanine ammonia-lyase (PAL) enzyme activities

The results showed that PAL activity was different in all treatments in the third and fourth weeks after cultivation of plants. Maximum activity in the third and fourth weeks could be

Table 2. Mean comparison of peroxidase enzyme activities in tomato plants treated with *B. bassiana* strains.

Treatment	Peroxidase enzyme activity (µmol H_2O_2 decomposed per mg protein per minute	
	Third week*	Fourth week*
TS7 (10^7 cell/ml)	0.346 abcd**	0.193 b
TS7 (10^9 cell/ml)	0.186 bcd	Not measured
TS12 (10^7 cell/ml)	0.109 cd	0.566 b
TS12 (10^9 cell/ml)	0.352 abcd	0.257 b
KJ24 (10^5 cell/ml)	0.492 ab	1.524 a
KJ24 (10^7 cell/ml)	0.435 abc	2.274 a
KJ24 (10^9 cell/ml)	0.602 a	0.506 b
Non-infected control	0.296 abcd	0.082 b
Rhizoctonia-infected control	0.080 d	0.113 b

* After cultivation of plants **Means followed by the same letters in a column are not significantly different.

Table 3. Mean comparison of phenylalanine ammonia-lyase activities in tomato plants treated with *B. bassiana* strains.

Treatment	Enzyme activity (mg per liter cinnamic acid in mg protein in minutes)	
	Third week*	Fourth week*
TS7 (10^7 cell/ml)	3.003 b**	1.776 d
TS7 (10^9 cell/ml)	1.42 e	Not measured
TS12 (10^7 cell/ml)	2.57 c	1.896 d
TS12 (10^9 cell/ml)	1.23 f	1.070 f
KJ24 (10^5 cell/ml)	1.82 d	1.540 e
KJ24 (10^7 cell/ml)	3.56 a	1.563 e
KJ24 (10^9 cell/ml)	2.86 b	2.943 a
Non-infected control	2.60 c	2.113 c
Rhizoctonia-infected control	1.45 e	2.343 b

*After cultivation of plants ** Means followed by the same letters in a column are not significantly different.

Table 4. Mean comparison of amount of total phenolic compounds in tomato plants treated with *B. bassiana* strains.

Treatment	Amount of total phenolic compounds (mg gallic acid per gr fresh weight of the plant)	
	Third week*	Fourth week*
TS7 (10^7 cell/ml)	16.27 d**	8.12 de
TS7 (10^9 cell/ml)	8.82 h	Not measured
TS12 (10^7 cell/ml)	22.82 c	8.80 d
TS12 (10^9 cell/ml)	33.49 a	7.69 e
KJ24 (10^5 cell/ml)	27.13 b	19.10 a
KJ24 (10^7 cell/ml)	13.02 f	10.43 c
KJ24 (10^9 cell/ml)	14.76 e	18.39 a
Non-infected control	9.59 h	18.94 a
Rhizoctonia-infected control	11.12 g	12.71 b

*After cultivation of plants ** Means followed by the same letters in a column are not significantly different.

measured in plants treated with isolate KJ24 at concentrations of 10^9 and 10^7 spores/ml, respectively, but plants treated with TS12 at the concentration of 10^9 spores/ml had the minimum activity of this enzyme at both time points. In the fourth week, the level of PAL activity in all plants – except from those treated with isolate KJ24 at the concentration of 10^9 spores/ml – was significantly reduced, compared with the infected and non-infected control plants (Table 3).

Total phenolic compounds

At the third and fourth weeks after the cultivation of plants, the amount of total phenolic compounds was significant in all of the treated plants. Maximum and minimum amounts of phenolic compounds were produced at the third week by the isolates TS12 and TS7, respectively at the concentration of 10^9 spores/ml. Also, maximum and minimum amounts of phenolic compounds at the fourth week were observed in the plants treated with isolates KJ24 (10^9 spores/ml) and TS12 (10^5 spores/ml), respectively (Table 4).

Discussion

It is likely that more than one antagonistic mechanism is operative in suppression of plant diseases by *B. bassiana* which include the production of antibacterial and antifungal secondary metabolites (Ownley et al. 2010; Azadi et al. 2015a). According to Azadi et al. (2015b), mycelial growth of *R. solani* was inhibited by volatile compounds and culture filtrates containing secreted metabolites of isolates TS12, TS7 and KJ24 of *B. bassiana*. Also in this study, isolates TS12 and TS7 were able to produce toxins in methylene blue agar medium. Certain toxins as well as antifungal and antibacterial compounds produced by microorganisms can be involved in the biocontrol activities as important factors (Harman 2000). The occurrence of the phenomenon of antibiosis by *B. bassiana* against plant pathogens including *Gaeumannomyces graminis* var. *tritici*, *Armillaria mellea*, *Rosellinia necatrix*, *Fusarium oxysporum*, *Botrytis cinerea*, *Pythium ultimum* and *R. solani* (Ownley et al. 2010) have been reported. Despite the production of toxins and secondary metabolites by isolates of *B. bassiana* they are not able to cease the growth of pathogens and create halos of inhibition in dual culture (Azadi et al. 2015a; Griffing 2007). Evaluation of chitinase enzyme activities showed that all isolates were able to produce chitinase enzymes in the presence of *Rhizoctonia* cell wall as the only carbon source and it is noted that the secretion of chitinase enzymes increased significantly on the fourth day, which is consistent with previous reports (Mustafa and Kaur 2010). It was also found that the amount of chitinases increased in various isolates of *B. bassiana* on the fourth and sixth days after inoculation. In other studies, chitinase production increased in various isolates of *B. bassiana* on the third day after inoculation. Although the amounts of chitinases produced by various isolates of *B. bassiana* are different and influenced by the carbon source and the test situation, the maximum of chitinase enzyme activities can be seen at 72 to 120 hours after inoculation (Petlamul and Prasertsan 2012).

Innate traits of the applied strains, as well as nutritional combinations of the media, carbon sources, and concentration, and carbon:nitrogen ratios are known to influence the growth and conidiogenesis of fungi (Petlamul and Prasertsan 2012). Therefore the growth rate and sporulation of every strain are different. The results showed that the presence of nitrogen in the SDA medium accelerates the growth of isolate TS12; while the growth rate of TS7 and KJ24 isolates was high on

PDA. Pandit and Som (1988) suggested that PDA can be used for the culturing of *B. bassiana* (Senthamizhlselvan et al. 2010), while some others successfully used SDA medium for the mass culturing of *Metarhizium anisopliae* and *B. bassiana* (Petlamul and Prasertsan 2012). In terms of sporulation, all isolates were able to produce more spores in SDB medium than in PDB medium. According to the studies carried out by Sharma et al. (2002), the better growth and sporulation of *Beauveria* spp. in the SDB medium may be due to the presence of peptone as a source of nitrogen (Senthamizhlselvan et al. 2010). Thus the presence of nitrogen in the SDA medium may accelerate the growth of isolate TS12. Similarly as in the natural environment, the amount of carbon and nitrogen in soil can affect the growth and sporulation, colonization, and the use of appropriate biocontrol mechanisms by different isolates.

For the evaluation of the mechanisms involved in the control of damping-off, enzyme activities, phenolic compounds and growth features were investigated. Previous studies have shown that some enzymes are related to ISR, including peroxidase and polyphenol ammonia-lyase. These enzymes cause the release of molecules involved in pathway signaling defense mechanisms of plant, and produce compounds such as phenolics (Alves Silva et al. 2004). Our results showed that the peroxidase activity increased in some treatments in the fourth week in comparison to the third week after cultivation of plants. The rapid increase of this enzyme activity followed by its reduction during the plant's defense responses is related to the generation of reactive oxygen species (ROS), which act as signal molecules to activate defense responses (Yang et al. 2014). Therefore, the amount of peroxidase and ROS increases rapidly under pathogen attack; but the high concentrations of ROS are toxic to the plant itself and cause cell damage. To control the level of ROS and to protect cells under stress conditions, plants produce ROS scavenging enzymes such as peroxidase to reduce ROS (Tsai 2011). Rapid increase of peroxidase enzyme activities in the third week after the treatments (*e.g.*, with TS7 at 10^7 spores/ml) is likely due to the activation of defense mechanisms. The activity of peroxidase enzyme in isolate KJ24 at 10^5 and 10^7 spores/ml increased in the fourth week after cultivation of plants. Although the activity of this enzyme was higher than in case of the infected control plants, because of the delay in the activation of plant defense responses, it was not able to control the disease in proper time and percentage of damping-off was high. Therefore the extent and speed of activation of defense responses for the inducing of resistance against the pathogens is important and vital (Ku and Preisig 1984).

Based on the results from measurement of plant phenolic compounds, the amount of these compounds increased in the third week and then reduced in the fourth week in all plants treated with fungal isolates (except those treated with KJ24 at 10^9 spores/ml), which represents the activation of plant defense responses by the treatments, particularly by TS12 and TS7 at the concentration of 10^7 spores/ml (34.78% damping-off). Our results are consistent with the findings of Madhavan et al. (2011). Increasing plant resistance is associated with increased phenolic compounds (Velazhahan and Vidhyasekaran 1994). In plants treated with KJ24 at the concentration of 10^9 spores/ml with 21.73% damping-off, the level of these compounds were high in comparison to infected control plants, but defense responses were not activated because these compounds increased gradually. Production of phenolic compounds and their concentrations are among the most important defense reactions of plants against pathogens. Under rapid damage of the cells, phenolic compounds are released quickly, which effectively prevents the penetration of pathogens; otherwise, under slow release of the components, it may let the rapid spread of diseases and quick development of symptoms.

Additionally, results showed that the activity of the PAL enzyme increased in the fourth week in comparison to the third in the case of all isolates except for KJ24 at the concentration of 10^9 spores/ml. Although this rapid increase can be indicative of defense mechanism activation, in some treatments including TS12 and TS7 at the concentration of 10^9 spores/ml, the amount of this enzyme was lower than those of the infected and non-infected control plants. PAL is one of the most important enzymes in the activation of defensive responses (Alves Silva et al. 2004), leading to increased phenolic compound production in response to pathogens (Parr and Bolwell 2000). Based on the above mentioned results, the gradual increase in PAL enzyme and phenolic compounds in KJ24 treatment at 10^9 spores/ml concentration cannot be the reason for the activation of defense responses; therefore the control of damping-off by this treatment was probably not due to the stimulation of induced systemic resistance, and it seems that another mechanism is involved in the reduction of disease by this treatment.

Lignification of the walls and its strengthening were done by H_2O_2 in the early stages of growth and defensive responses (Yang et al. 2014). Considering the role of peroxidase enzymes in the generation of ROS, increased production of H_2O_2 is associated with the increase of peroxidase activities. After passing through the stage of maturity and reducing the amount of H_2O_2, the amount of peroxidase enzymes also decreases. In the uninfected control plants, the amount of peroxidase enzymes increased in the third week after cultivation of plants. However, this increase was less than in the case of the treated plants. Studies carried out by Ippolito and Nigro (2000) also showed that the amount of peroxidase enzymes increased in the uninfected control fruits, however, it was less than that of the fruits treated by antagonist *Aureobasidium pullulans*. So, rapid increase in peroxidase enzyme, as well as the increase in growth factors in KJ24 treatment at 10^9 spores/ml have probably been due to plant growth stimulation for the passage

of the initial phase. Plant growth stimulation by antagonists plays an important role in the protection of plants against root diseases. With the growth stimulation of the plant, the infected roots are replaced quickly by new roots; the result caused the passage of seedlings from the susceptible stage and the reduction of disease damage. Harman et al. (2004) found that the existence of such activity by entomopathogenic fungi is similar to the activity of plant growth promoting rhizobacteria. In TS12 and TS7 at the concentration of 10^7 spores/ml (34.78% damping-off), the activity of PAL, and the amount of phenolic compounds in the third week showed a rapid increase in comparison to the fourth week. Therefore, it seems that an increase in this enzyme under the mentioned treatments may be due to the activation of induced systemic resistance; and, probably, treatments with TS7 and TS12 at the concentration of 10^7 spores/ml are able to reduce damping-off, and their effective control is due to the plant resistance stimulation.

In the case of isolate TS7 the induction of plant resistance could be due also to its rapid and abundant sporulation. The spores could act as a stimulant and cause the activation of the plant defensive responses. Based on *in vitro* studies, isolate TS12 demonstrated the same behavior as KJ24 (Azadi et al. 2015b), but in the greenhouse they were different. This behavior can rise from differences in the growth pattern of two strains. Based on the results, this strain had high growth rate on the SDA medium (nitrogen rich). It is likely that because of the nitrogen shortage in the soil, this strain cannot grow well and colonize the plant, but it can induce plant resistance by producing high number of spores. Induction of resistance along with other biocontrol mechanisms such as competition and antibiosis can be effective in plant protection against plant pathogens. Since the proper functioning of antagonists against soil-borne pathogens is related to environmental conditions, soil, and population of antagonists, it seems that the difference in the behavior of antagonists and the type of mechanisms used to control damping-off by isolates of *B. bassiana* is dependent on its compatibility with the existing conditions.

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ARTICLE

Valorization of wheat bran for cost-effective production of cellulolytic enzymes by *Aspergillus fumigatus* SKH2 and utilization of the enzyme cocktail for saccharification of lignocellulosic biomass

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ABSTRACT Production of cellulolytic enzymes like CMCase (endoglucanase), FPase, and xylanase by *Aspergillus fumigatus* SKH2 under solid state fermentation was carried out employing wheat bran as low cost substrate. Fermentation time, medium pH and incubation temperature were optimized at 48 h, pH 5.0 and 35 °C, respectively. At optimized state, CMCase (endoglucanase), FPase and xylanase of 826, 102 and 1130 U/gds yield was noticed, respectively. Crude enzyme cocktail was assayed at varied pH and temperature, and pH 5.0 and 35 °C were proved to be optimal for the studied enzyme activities. Fourier transform infrared spectroscopic FTIR analysis attested that NaOH was a good delignifying agent for sugarcane bagasse and grass *Aristida* sp., which enhanced subsequent saccharification efficiency of cellulolytic enzyme cocktail. By correlating FTIR analysis with saccharification profile it was found that highest saccharification was achieved after 16 h and 48 h after treating with 1M and 3M NaOH for sugarcane bagasse and *Aristida* sp., respectively. The present investigation validates eco-friendly and cost effective production of *A. fumigatus* SKH2 cellulolytic enzyme cocktail using agricultural waste materials, and subsequent application of this cellulase mixture for saccharification of lignocellulosic biomass, which collectively endorse the employment of the bioprocess to produce biofuel in future.

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KEY WORDS

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fermentation
lignocellulosic biomass
saccharification

Introduction

From the last few years, there has been an increasing research importance in the worth of lignocellulosic biomass. These are the maximum promising feedstock as natural and renewable reserve. Among many of the developing countries, it's a routine practice that such agricultural wastes are not been fully castoff and then have turn out to be a major cause of environmental pollution (Soudham 2015). Naturally, hemicellulose, cellulose and lignin are the major constituents of plant cell walls and among all of them, cellulose is the most conjoint and profuse component of all plant matter.

Since the last decade, demand for some industrially important enzymes increases and among them cellulases are pioneer. Cellulase is being employed in many industrial applications mainly but not limited in the field of cotton processing, paper salvaging, agronomy and in the field of research and development (Dong et al. 2015). Besides, the production of fuel ethanol from lignocellulosic biomass through cellulase hydrolysis is a most promising tool of the modern world. The most efficient technology for the conversion of the lignocellulosic biomass to fuel ethanol is based on the enzymatic breakdown of cellulose using cellulolytic enzymes (Das et al. 2013).

India is an agricultural land that produced a large magnitude of lignocellulosic wastes which can be utilized for production of useful industrial enzymes or enzyme-based products. Enzymatic hydrolysis of such agricultural wastes provides an ecofriendly means of depolymerizing cellulose and other carbohydrates at high yields (Das et al. 2013). But the most important barrier to produce low cost fermentable sugar from biomass is that the cellulose component of lignocellulosic materials is tightly bound to hemicelluloses and lignin. Lignin protects from degradation and confers hy-

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drolytic stability and structural robustness to the cell walls of the plants. To stunned lignocellulose resistance, pretreatment is required to alter the structure as well as its submicroscopic chemical composition, so that enzymatic hydrolysis of the carbohydrate fraction to monomeric sugars can be achieved more quickly with greater yields. Till date, the most widely familiar pretreatment techniques include liquid hot water, steam explosion, treatment with alkali, dilute acid, sulphur dioxide, hydrogen peroxide, organosolve, ammonia fiber explosion (AFEX), CO_2 explosion, ionic liquids etc. (Jena et al. 2016). Considering these, alkali, acid and peroxide treatments are widely applied in industries, but the suitability and the concentration of the chemicals depend on the type of substrate utilized (Chiaramonti et al. 2011).

Literature review attested that cellulolytic filamentous fungi belonging to the genus *Trichoderma* have widely been considered for production of cellulolytic enzymes and also preferred by many companies worldwide (Kovács et al. 2009; Sørensen et al. 2013; Rani et al. 2014). However, wild types *T. reesei* is generally characterized as high endoglucanase and cellobiohydrolase producer, but it secretes the β-glucosidase in minimum quantity and thus, additional supplementation of β-glucosidase is need to carry out saccharification of cellulosic substrates efficiently (Lynd et al. 2002; Rani et al. 2014). Generally, the production of cellulases accounts for about 40% of total cost in bioethanol production (Gray et al. 2006). To minimize the production cost, attempt should be taken for utilization of zero-valued/low cost easily available lignocellulosic materials for the production of cellulolytic enzymes. Moreover, the microorganisms capable to utilize the complex lignocellulosic materials also liberates other hydrolyzing enzymes like xylanase, ligninase etc. along with cellulolytic enzymes. Presence of these accessory enzymes enhances the biodegradation of the complex substrates. So, the approach for production of crude enzyme cocktail from low cost substrates and its subsequent application in saccharification is more viable than commercially available cellulase due to their reasonable cost and presence of other accessory enzymes in the crude preparation which enhances the saccharification efficiency.

The solid-state fermentation (SSF) can be of special interest in bioprocesses where crude fermented product could be used directly as enzyme source. This technology is most efficient, because this processes requires lower energy, high product concentration, lower input of infrastructure and skill, produce less wastewater and are ecofriendly as they resolve the problem of solid waste disposal (Halder et al. 2016). Currently, industrial demand for cellulases is being met by production methods using submerged fermentation (SmF) processes. The cost of production through SmF systems is, however, very high and uneconomical. Therefore, it is necessary to reduce the production cost by deploying alternative production methods such as SSF. In the arena of cellulase production and saccharification for cost-effective bioethanol production, lingo-cellulosic biomass may contributes significantly as substrate which replenished constantly in nature, available as wastes in the form of pre- and post-harvest agricultural losses as well as wastes of food processing industries and therefore considered as world's largest reusable reservoir of potentially fermentable carbohydrates (Begum and Alimon 2011; Pandey 2015).

Among the various types of lingo-cellulosic biomass, wheat bran was employed as substrate for production of cellulolytic enzymes, whereas sugarcane bagasse and grass *Aristida* sp. were employed for generation of fermentable reducing sugar through saccharification. Sugarcane bagasse is the residual biomass (fiber) waste left after juice extraction while *Aristida* is abundant in the local area and remain unutilized. It also has nearly cosmopolitan distribution and rarely reported to be used as substrate for saccharification but have immense potential to be used for the same.

Thus, the aims of the current study were to optimize the production of low cost cellulolytic enzymes through solid state fermentation by fungal isolate *Aspergillus fumigatus* SKH2 and optimization of production of fermentable reducing sugar by enzymatic saccharification of the pretreated sugarcane bagasse and *Aristida* grass for better yield.

Materials and Methods

Microorganism cultivation and inoculum preparation

Aspergillus fumigatus SKH2 was obtained from the culture collection of Department of Microbiology, Vidyasagar University (West Bengal, India). The strain was grown on potato dextrose agar (PDA) slants at 30 °C for 5 days until good sporulation occurred and stored at 4 °C until use. Inoculum was prepared by adding 5 ml of sterile distilled water containing 0.1% Tween 80 to a fully sporulated culture slants. The spores were dislodged by gentle pipetting under strict aseptic conditions and the number of spores in the suspension was determined using a Neubauer chamber. The concentration of the spore suspension was adjusted ~5 $\times 10^9$ spores/ml. Then the spore solutions were collected in a sterilized bottle and stored in refrigerator for future use. For detection of cellulolytic activity in agar plate, the spore suspension was plated on Mandels's agar media [having the composition of KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.4; urea, 0.3; $MgSO_4 \cdot 7\ H_2O$, 0.3; $CaCl_2 \cdot 2H_2O$, 0.4; $FeSO_4 \cdot 7\ H_2O$, 0.005; $MnSO_4 \cdot H_2O$, 0.0016; $ZnSO_4 \cdot 7\ H_2O$, 0.0014; $CoCl_2$, 0.0020; carboxymethyl cellulose (CMC), 10; and agar 17.5 g/l; pH 5.0]. After 3 days, the plates were flooded with Congo red solution (1%), after 5 min it was discarded, and the plates were washed with

1 M NaCl solution allowed to stand for 15 min. The clear zone around the colony indicates the cellulolysis. Cellulase activity on CMC agar was recorded as the Index of Relative Enzyme Activity (I_{CMC}) which is a ratio of clear zone diameter / colony diameter.

Optimization of cellulolytic enzymes production

Wheat bran (WB) was procured from local market in Midnapore (West Bengal, India). Cellulolytic crude enzyme cocktail was produced by using WB as substrate through solid state fermentation (SSF). SSF was carried out in 250-ml Erlenmeyer flask containing 5 g of WB moistened with modified Mandels's salt medium (Mandels et al. 1976) composed of $(NH_4)_2SO_4$ 1.4%, KH_2PO_4 2.0%, $CaCl_2$ $2H_2O$ 0.4%, $MgSO_4$ 0.3%, peptone 1.0%, NaCl 0.3%, $FeSO_4$ 0.005%, $MnSO_4$ 0.0016%, $ZnSO_4$ $7H_2O$ 0.0014%, and $CoCl_2$ 0.002%. The resultant mixture had an initial moisture content of 50%. Sterilized medium in the flask was homogeneously mixed with 0.5 ml spore suspension, and then it was subjected for fermentation. Effects of pH, temperature and fermentation time were optimized by one variable at a time (OVAT) approach.

Extraction and condensation of cellulolytic enzymes

After fermentation, enzymes were extracted with sterile distilled water (1:10 substrate to volume ratio) by shaking for 1 h at 120 rpm, followed by centrifugation at 5000 g. The supernatant was used as crude enzyme source. The crude enzyme solution was condensed by rotary evaporator (Eyela, Japan).

Optimization of pH and temperature of crude enzyme activity

To determine the optimum cellulolytic condition, activity of the different cellulolytic enzymes was checked at varied pH and temperature. To determine the pH optimum of the cellulolytic activity, citrate buffer (pH 3, 5, and 6), phosphate buffer (pH 7) and TRIS buffer (pH 8, 9) in 0.05 M concentration were used. Optimum temperature for the activity was determined by incubating the crude enzyme mixture at varied temperature conditions (20, 30, 40, 50, and 60 °C).

Pretreatment of lignocellulosic biomass

Grass *Aristida* sp. and sugarcane bagasse were collected from the adjoining area and market of Midnapore town (West Bengal). The residues are washed, dried and cut into small pieces (~5 mm in diameter). Alkaline (NaOH), acidic (H_2SO_4), oxidizing (H_2O_2) agent mediated pretreatments were carried out to make the substrate more accessible to enzymatic saccharification. Briefly, grass samples and sugarcane bagasse (10%, w/v) were boiled with sulphuric acid (1-5 M), sodium hydroxide (1-5 M) or hydrogen peroxide (1-5%, w/v) for 30 min. After cooling, the samples were washed with tap water and air dried.

Enzymatic saccharification

Untreated (native) samples, and the water-insoluble residue of pretreated grasses and sugar cane bagasse were subjected to enzymatic hydrolysis (substrate:enzyme:buffer = 1:2:3). Enzymatic saccharification was performed in a rotary shaker at 100 rpm up to 48 h. Samples were withdrawn periodically and the amount of reducing sugar released was estimated colorimetrically by dinitrosalicylic acid method (Miller 1959).

Enzyme activity assays

Endoglucanase or CMCase (E.C. 3.2.1.4) activity

Endoglucanase or CMCase (E.C. 3.2.1.4) activity was determined by incubating 0.5 ml of enzyme solution with 0.5 ml of 1.0% (w/v) CMC (carboxymethyl cellulose) (Sigma, St. Louis, MO, USA) under buffered conditions (Das et al. 2013). After incubation at optimum temperature for 30 min, the reaction was stopped by adding 1 ml of 3,5-dinitrosalicylic acid reagent. Liberated reducing sugars were estimated colorimetrically according to the method of Miller (1959) using glucose as standard.

Filter paper degrading activity (FPase)

Filter paper degrading activity (FPase) was determined using processed Whatman No 1 filter paper as substrate (50 mg/ml) according to the method of Wood and Bhat (1988). After incubation at 50 °C for 30 min, the reactions were stopped by adding 1 ml of 3,5-dinitrosalicylic acid (DNS) reagent. Liberated reducing sugar was estimated colorimetrically according to the method of Miller (1959) using glucose as standard.

Xylanase (E.C. 3.2.1.8) activity

Xylanase (E.C. 3.2.1.8) activity was determined by measuring the release of reducing sugars from 1% (w/v) birch wood xylan under optimum assay condition (Bailey et al. 1992). Free sugar released was analyzed by the DNS method. One unit of enzymatic activity was defined as the amount of enzyme required to produce 1 µmol of reducing sugars (measured as monosaccharide content) by hydrolyzing respective raw substrate per minute under specified assay conditions.

Fourier Transform Infrared Spectroscopic of native and pretreated biomass

The infrared spectral analysis of the untreated and treated grasses and sugar cane bagasse were studied using Fourier Transform Infrared Spectroscopy (Schimadzu, Japan), and the Essential FTIR (Operant LLC) software was used to record and analyze the FTIR data. Biomass of 3 mg was dispersed in 300 mg of spectroscopic grade potassium bromide (KBr) and subsequently pressed into disks at 10 MPa for 3 min (Binod et al. 2012). The spectra were obtained in transmission mode with an average of 30 scans and a resolution of 4 cm^{-1} in the range of 4000-400 cm^{-1}.

Results and Discussion

Currently, the researchers are paying more interest to the utilization of plant-derived waste residues. In this study, we have chosen sugarcane bagasse and grass *Aristida* sp. for this purpose because they are arisen as plant-waste annually in high amount. These wastes consist of lignin and complex polysaccharides (*e.g.*, cellulose, hemicellulose), which can be converted into fermentable sugars by pretreatment and saccharification processes. For cost effective production of fungal cellulolytic enzymes, wheat bran can be considered as good supporting substrate probably due to the presence of various available nutrients, good porosity, suitable particle size and consistency required for fungal anchorage and enzyme excretion (Sun et al. 2008; Kar et al. 2013). Its texture remains loose even in moist condition, thereby provides a large surface area and increased water holding capacity (Das et al. 2013). Considering these properties, wheat bran was applied as substrate in this study to produce cellulolytic enzymes in high yield using the cellulolytic fungal isolate *Aspergillus fumigatus* SKH2. The fungal strain was previously isolated from paper mill effluent and maintained in slant culture. During the evaluation of cellulolytic activity in CMC containing agar plate, I_{CMC} value (clear zone diameter / colony diameter) of 1.69 was estimated after 3 days of incubation. The result revealed that the fungal strain has the potentiality to produce cellulolytic enzymes which degrade the CMC around the colony.

Production of crude A. fumigatus SKH2 cellulases and xylanases

Optimization of fermentation conditions

For enhancement of productivity of any microbial metabolite in laboratory, the levels of the physico-chemical factors influencing the bioprocess should be optimized. The optimization and designing of fermentation conditions is an important footstep to develop economically feasible bioprocesses. In this context, solid state fermentation (SSF) has been considered as the cheapest and environment-friendly approach. In the present study, wheat bran substrate moistened with Mandel's mineral salt solution (Mandels et al. 1976) was used as fermentation medium, and the physical conditions like fermentation time, medium pH and incubation temperature were optimized sequentially following one variable at a time approach (OVAT) to enhance the production yield of *A. fumigatus* SKH2 cellulolytic and xylanolytic enzymes (CMCase, FPase and Xylanase).

Production of cellulolytic enzymes increased during the first phase of SSF, and the highest yield was obtained at the 48th h of fermentation (Fig. 1a). Enzyme yield was decreased after 48 h which may be due to nutrient depletion, toxic end products accumulation, change in medium pH or loss of moisture in the medium (Hosamani and Kaliwal 2011).

Growth, development and metabolic activities of an organism are significantly influenced by the temperature used for incubation. Hence, it is essential to optimize this parameter for maximal cellulolytic production. As can be seen on Figure 1b, the production of *A. fumigatus* SKH2 CMCase, FPase and xylanase under SSF condition was highest at 35 °C. The low enzyme yield below this temperature is possibly due to alteration of membrane permeability which restricts flow of nutrients inside the cells (Iftikhar et al. 2008; Halder et al. 2014). On the contrary, at higher temperature, the maintenance energy requirement of cellular growth was high due to thermal denaturation of the enzymes of the metabolic pathway, resulting in lower production of the metabolites (Das et al. 2013; Dutta and Kumar 2014). At optimized state, 826, 102 and 1130 U/gds maximal yields were noticed for the CMCase (endoglucanase), FPase and xylanase enzymes, respectively.

The production yield of the studied cellulolytic enzymes was higher at slightly acidic pH; the best yield could be achieved at pH 5 (Fig. 1c). Enzyme production decreased at alkaline pH which probably due to the proteolytic inactivation of the cellulose (Dutta and Kumar 2014). Our results showed that slightly acidic conditions supported the cellulase and xylanase production, while at higher pH, the activities decreased gradually. It is supposed that the H^+ concentration in the fermentation medium had a significant effect on the enzyme production. The optimal pH for cellulase production from *Aspergillus niger* and *Aspergillus phoenicis* were reported between 6.0-7.0 and 4.5-4.8, respectively (Akiba et al. 1995; Yeoh et al. 1986).

Temperature and pH optimum of the crude enzyme activities

Temperature and pH are key factors that affect the catalytic

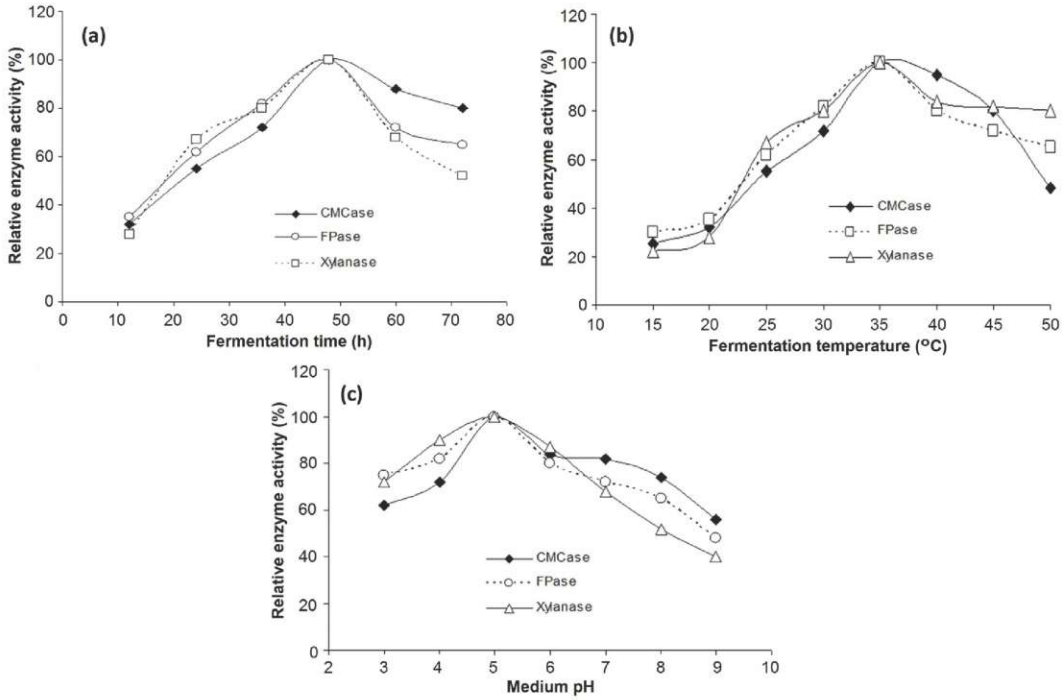

Figure 1. Effect of fermentation time (a), temperature (b) and medium pH (c) on production of cellulolytic enzymes by *Aspergillus fumigatus* SKH2 during SSF.

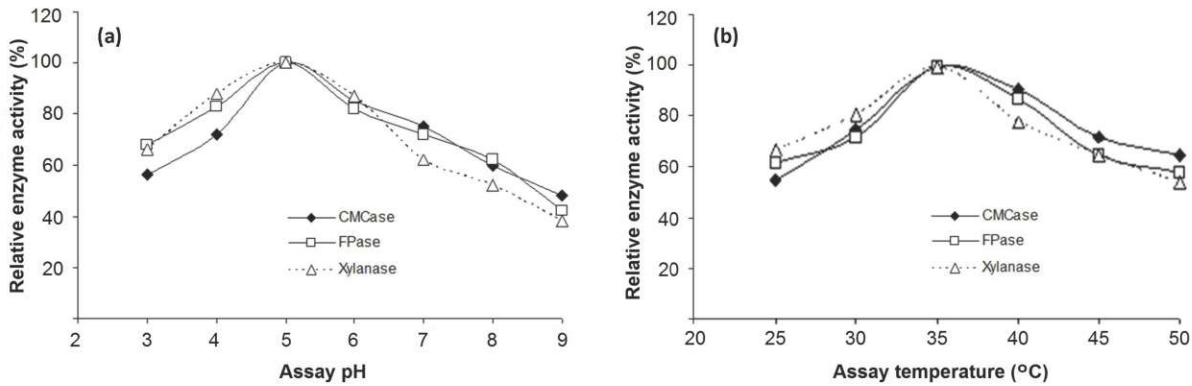

Figure 2. Effect of pH (a) and temperature (b) on activity of cellulolytic enzymes of *Aspergillus fumigatus* SKH2.

efficacy and stability of an enzyme, hence, for practical applications, determination of the optimal pH and temperature conditions for the activity is obligatory. Here we assayed the cellulolytic and xylanolytic activities in the *A. fumigatus* SKH2 crude enzyme preparation in different pH and temperature conditions. It was found that all three studied enzymes (CMCase, FPase and xylanase) were stable in the pH range of 4-6. The optimal pH condition for their activity was around pH 5 (Fig. 2a). It may be due to the acidic nature of the enzymes and abundance of acidic amino acid at the active site, which undergoes protonation and deprotonation by the influence of environmental pH and in turn regulate the enzyme activity (Jana et al. 2013).

Stability of cellulases and xylanases in a temperature range is one of the important parameters which determine their application in various industrial processes. In the present study, all three investigated crude enzymes retained more than 70% of their original activity between 30-45 °C presenting maximal activity values at 35 °C (Fig. 2b).

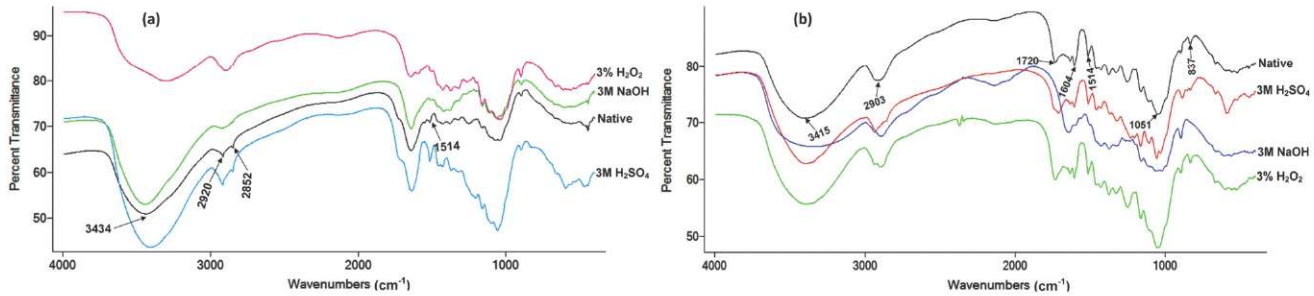

Figure 3. Comparisons of FTIR spectra of pretreated grass *Aristida* sp. (a) and sugarcane bagasse (b) with their native (untreated) form.

Saccharification of sugarcane bagasse and grass samples

Pretreatment and delignification

It is known that the lignin content of a plant-derived residue may affect the action of cellulolytic and xylanolytic enzymes which is required to achieve higher fermentable sugar yields from the substrate. Therefore, we tested oxidizing (H_2O_2), acidic (H_2SO_4) or alkaline (NaOH) agents in varied concentrations to delignify the substrates. After treatment, FTIR analysis was performed to find out the best agents for delignification. Figure 3 shows the comparison of FTIR spectra of native (untreated) and treated (in 3 M or 3% concentrations) grass (Fig. 3a) and sugarcane bagasse (Fig. 3b) samples.

The absorption band of 3434 cm^{-1} assigned for -OH stretching indicates the alcoholic and phenolic hydroxyl groups in the lignin polymer (Fig. 3a). The absorption was reduced after H_2O_2 and NaOH treatment in contrast with untreated grass (Fig. 3a). This is may be due to de-lignification which subsequently increased accessibility of cellulose by the enzymes. The C-H stretching vibrations of methoxyl group of lignin were assigned to 2920 and 2852 cm^{-1} (Hergert 1960); these distinct peaks were disappeared after NaOH and H_2O_2 treatment. The semi-circle stretch of para-substitute benzene rings was assigned to 1514 cm^{-1} (Adapa et al. 2011). This peak was disappeared after NaOH treatment indicating the removal of aromatic benzene rings from lignin. Moreover, the intensity of this band decreased after H_2SO_4 and H_2O_2 treatment which also indicates benzene ring rupture.

In the case of sugarcane bagasse, the intensity of absorption band at 3415 cm^{-1} represents the stretching of –OH groups. The absorption at 3415 cm^{-1} was increased after H_2O_2 treatment which indicated that partial hydrogen bonds within and between cellulose fibers were destroyed. On the contrary, opposite result was found after NaOH treatment which is in accordance with the results of grass.

Carbonyls as important functional groups mainly occur in the side chains of lignin structural units. These aldehyde and keto groups are lying in C-γ or C-β position, respectively. The disappearance of spectral band at 1720 cm^{-1} assigned to the carbonyl (C=O) stretching of unconjugated ketones indicated that the side chain of lignin was also broken down during NaOH and H_2SO_4 treatment and removal of lignin (Bykov 2008; Das et al. 2013). The small absorption bands of native form at 1604, 1514, 1051 and 837 cm^{-1} represents typical lignin structures (Bykov 2008; Adapa et al. 2011). The absorption band at 1514 cm^{-1} assigned to semi-circle stretch of para-substitute benzene rings disappeared after NaOH treatment, which indicates the removal of aromatic benzene rings from lignin polymer. After H_2SO_4 and H_2O_2 treatment, the intensity of this band decreased which suggests the rupture of the benzene rings (Adapa et al. 2011). The bands at 1604 and 1051 cm^{-1} are attributed to C-Ph and O-H stretching of primary alcohol vibration, respectively. These bands are generally found in the lignin aromatic structure. After pretreatment with NaOH and H_2SO_4, these bands are changed or disappeared.

Anyway, our results indicate that NaOH is better delignifying agent than the other compounds tested when grass

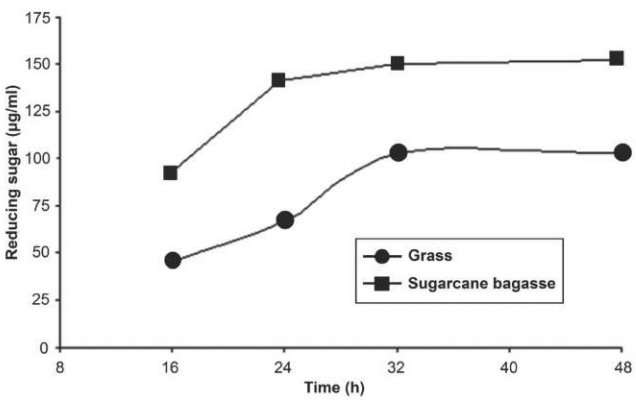

Figure 4. Time dependent saccharification of native grass and sugarcane bagasse by crude cellulolytic enzyme.

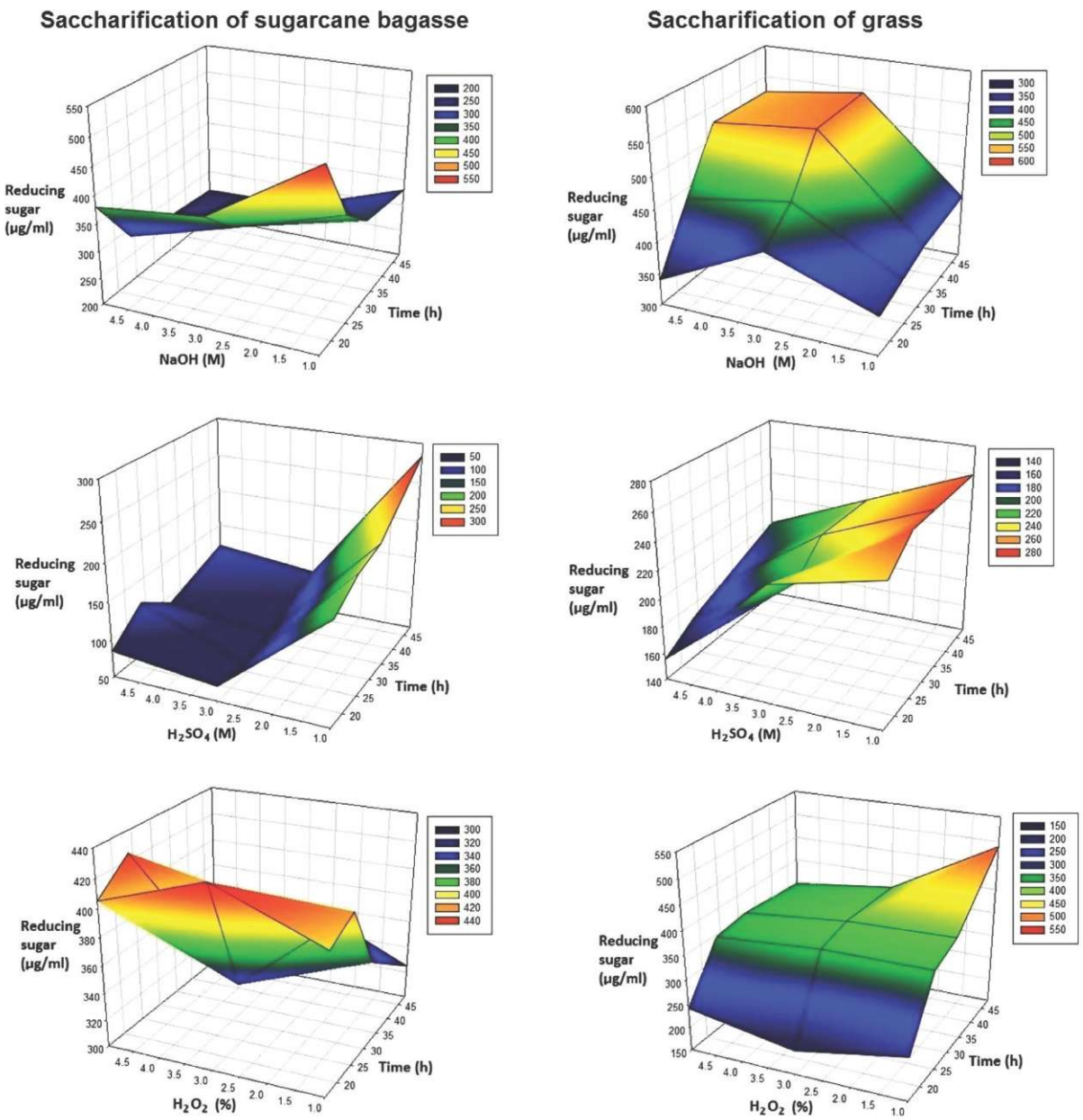

Figure 5. Dose and time dependent saccharification (reducing sugar yield) profile of lignocellulosic substrates treated with various agents under investigation.

(Aristida sp.) and sugarcane bagasse samples were used as substrate.

Optimization of pretreatment agent dose and treatment time on saccharification

Enzymatic hydrolysis of sugarcane bagasse and grass substrates in native and after pretreated form by crude enzyme cocktail (containing CMCase endoglucanase, FPase, and xylanase in 7.69:1:11.69 ratio) was carried out at 48 h. It was found that the sugar yield from both pretreated residues was varied in dose and time dependent manner. Untreated (native) samples from both residues were also subjected for saccharification, in which moderate increase in the reducing sugar content was detected (Fig. 4). It indicates that the crude enzyme preparation may contain some ligninolytic enzymes like lignin peroxidase, manganese peroxidase, laccase etc., which facilitate the action of cellulolytic enzymes.

In case of sugarcane bagasse, highest reducing sugar yield was achieved after 16 h incubation with the samples treated with 1 M NaOH. In grass samples, it was maximal with 3 M NaOH treated residues after 48 h incubation (Fig. 5). Based on these results, it can be concluded that NaOH is a useful agent to remove most part of the lignin polymer, thus, the subsequent saccharification process has been enhanced.

Conclusion

A reduction of the cost of fermentable reducing sugar production can be achieved by reducing the cost of raw materials. Using zero valued lignocellulosic materials such as agricultural residues, grasses, forestry wastes, sugarcane bagasse and other low-cost biomass can significantly reduce the cost of raw materials. Apart from that, reduction of the cost of cellulolytic enzyme production is also a key issue in the enzymatic hydrolysis of lignocellulosic materials. The above study theorized that *Aspergillus fumigatus* SKH2 is a potent cellulolytic fungal isolate which utilized wheat bran as robust anchorage cum substrate for production of cellulolytic enzymes like CMCase (endoglucanase), FPase, and xylanase and hence make the bioprocess proficient. Moreover, NaOH is found as good delignifying agent for sugarcane bagasse and grass *Aristida* sp., which enhances the saccharification efficiency of cellulolytic enzyme cocktail by many folds. As a whole, the present investigation validates eco-friendly and cost effective production of cellulolytic enzyme cocktail by *Aspergillus fumigatus* SKH2 from agricultural waste and subsequent application of the same for saccharification of lignocellulosic biomass. Considering the simplicity and proficiency, production of bioethanol from the generated reducing sugars by the action of yeast or bacteria in single or in combination will explore a new horizon in upcoming eons as an alternative sustainable approach to replenish the ever increasing demand of biofuels.

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ARTICLE

Effects of electric current frequencies, laser irradiation and combined treatment on *Saccharomyces cerevisiae* viability

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ABSTRACT In this research, we examined the influence of low voltage (9 V) electric current frequencies (1.4 and 17 Hz), laser irradiation (648 and 532 nm) and combined treatment (one frequency and one laser beam) on the viability of baker's yeast (*Saccharomyces cerevisiae*). Each treatment was conducted using modified methods and equipment in air-filter equipped working chamber. Staining was performed by a non-vital/vital staining technique that has shown an increase in viability of all samples. Counting of yeast cells in 1 ml of sample gave us several positive results in terms of different treatments, cell viability and increase in the number of healthy cells. Treatment with electric current at higher frequencies (4 and 17 Hz) showed increased cell death counts and, although compensating by an increase in viability, the 17 Hz frequency was considered more hazardous. The most adequate treatments (both increased viability and cell count) were the combined treatments (1 Hz/4 Hz + one of the two laser beams). Although, all electric treatments show certain increases in cell viability, combined treatments (1 or 4 Hz coupled with green or red laser beam) show the most promise in achieving both increased cell viability and increased cell counts.
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KEY WORDS

cell viability
electrical current
laser irradiation
Saccharomyces cerevisiae

Introduction

Problems in industrial mass production are concentrated mainly on low success rates in increasing biomass, while also maintaining healthy starting colonies. Due to high demand in this industry sector, there is a need to grow and maintain a high viability yeast culture (especially in large bioreactors with tens of thousands of liters in volume) (Babayan and Bezrukov 1985). Industrial treatments rely mostly on mechanisms discovered through scientific research based on treatment of baker's yeast with electricity or low wavelength electromagnetic radiation. (Karba et al. 1991; Piera V 1992; Yun et al. 1998; Wattanakaroon and Stewart 2000; Teixeira and Mira 2011). These findings point out that yeast cells initially divide swiftly and in great numbers, but then start to recede for unknown reasons as the electric treatment continues (Beschkov and Peeva 1994; Alvarez et al. 2003). According to several authors (Jacob et al. 1981; Blenkinsopp et al. 1992; Martin 1992; Thatipamala et al. 1992; Chi et al. 1995; Fologea et al. 1998; Wang et al. 2005), treating certain types of yeast with electric current leads to lower living and active cell counts. This is due to focus being placed mainly on higher voltages and duration of the treatment itself. Also, it was confirmed that a type of bioelectric effect occurs during treatment, which produces a lower viability ratio in the growth medium (Omori et al. 1996; Stewart et al. 1999; Bairva and Bastila 2010).

The main source of problems for researchers is the asynchronous yeast cell cycle, which increases active yeast cells and creates a negative nutrient feedback, so the desired effects actually become a setback (Rice and Ewell 2001). Problems arise when trying to achieve roughly 10% of healthy cells in the total biomass in larger industrial bioreactors (Peguin and Soucaille 1996). These authors emphasize that treatment with alternating electrical current at 20 V and frequency of 10 MHz may produce acceptable numbers of colony forming units. The issue of numbers of colony forming units persists, when the acceptable number of cells is between 4-9 x 10^8 units/ml, but only in special industrially modified growth mediums (Nakasono et al. 1997; Oppezzo and Pizarro 2001).

By adding experiments with laser irradiation treatment of living cells, we continue to add more effects and possibilities, along with new obstacles, to this work. Laser irradiation treatment schematics can be found in several research experiments (Niemann 1983; Matsunaga et al. 1984; Chen et al. 1998; Petin et al. 2001) where pulsating ultraviolet (UV) and stationary infrared (IR) radiation was used to achieve the phenomenon of photo-inactivation of baker's yeast and other

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microorganisms. These authors indirectly conclude, that a minimum dosage of 68 mJ/cm^2 during a 20 s treatment with a beam pulsating at the source represents a sufficient threshold to excite cells in various ways. Several authors already wrote about the effect of increasing cell viability through different types of irradiation (Blenkinsopp et al. 1992; Piera 1992; Stewart et al. 1999; Wang et al. 2005).

This research combines three different low voltage (9 V) frequencies (1.4 and 17 Hz) of electric current (alternating current - AC), two laser beams (648 and 532 nm, both at 5 mW) and blends these two treatments into a new, combined treatment. Furthermore, by adding the cell viability parameter to cell counts in 1 ml of sample we sought to summarize and visualize changes in treated and non-treated cells, as well as to investigate these methods for potential industrial use.

Materials and Methods

Material

For the purposes of this research we used baker's yeast (special industrial strain of *Saccharomyces cerevisiae*), which we took from a commercial product line (Di-Go© industrial product by Podravka-Croatia) due to its stability and industrial importance.

Prototypes and experimental equipment

The working equipment included several experimental prototype technologies such as:

1.) Modified prototype laser irradiation and combined treatment rectangular receptacles (two were made and each with a total volume of 10 ml; high temperature resistant; equipped with separate red and green Helium-Neon type lasers at wavelengths of 648 and 532 nm; final irradiance of 5 mW during 20 s = 100 mJ/cm^2; lasers were pointing downwards into the test receptacle).

2.) Electric current converter prototype for low voltage AC output with a choice of different frequencies (hand crafted to convert standard 220 V AC to a final output of constant 9 V with a range of selectable frequencies between 1 Hz - 115 kHz). Selected frequencies were conducted via two stainless steel electrodes (45 mm apart) placed on the sides of a purpose-made high temperature resistant receptacle (with a total volume of 360 ml).

3.) Modified working chamber with an air-filter (hand crafted to provide enclosed environment for aseptic type of work; air-filter was coated with several layers of active coal; hermetically sealed during treatments; clean and filtered air pumped inside via air-filter fan; rectangular in shape; total volume of 0.15 m^3).

Preparing the working yeast solution and experimental phase

We prepared a special type of industrial growth medium (working yeast solution - WYS) to act as a base industrial medium identical to those used for growing in bioreactors (Thatipamala et al. 1992). For samples we used a 1% solution of baker's yeast in the industrial growth medium (1.5 g of fresh yeast in 150 ml of industrial growth medium, this process was repeated as many times as needed) with continuous mixing on the magnetic mixing machine (1 min on 300 revolutions). Every experiment had a control series and a treatment series, although combined treatment had an additional control. During this research a total of 840 tests were done (every parameter test was repeated 30 times).

1.) *Low voltage electric current treatment*: 150 ml of WYS was treated with one of the three selected frequencies (1.4 or 17 Hz) for 15 min, while the control sample was placed in the incubation chamber (warm, dry, dark container with a constant temperature of 27 °C). Treated samples were placed next to the control sample during the waiting period (24 h).

2.) *Laser irradiation treatment*: From the WYS we took 10 ml of 1% yeast solution, transferred it to a modified receptacle attached to a laser, and irradiated the sample for 20 s. A control sample (10 ml) was taken and placed together with the treated sample in the incubation chamber for the 24 h waiting period.

3.) *Combined treatment*: For this treatment we applied one low voltage electric current treatment (and took additional control + sample for each of the frequencies) and then irradiated the sample with a laser for 20 s. After the treatments, the double-treated sample and control samples (both regular and additional control) were placed in the incubation chamber for the 24 h waiting period.

Microscopic analysis and gathering the data

We stained the samples with 1% methylene blue stain (Semikem d.o.o. BiH-cat. no. 26071 Metilensko Plavo). Cells were counted using a standard hemocytometer method in the Bürker-Türk counting chamber (DHC-B02 hemocytometer) using a light microscope (Olympus CH20). For photography and detailed analysis we used an advanced light microscope (Olympus BX41) with a mounted digital camera (DP12). For this purpose we used registered official software (Olympus DP12 Soft DP12-CB Ver.01.01.01.42.® Olympus Corp.).

Raw data was expressed as a counted number of cells (living and dead cells differentiated and incorporated into results) and then processed into percentage of viable cells (%), and number of cells in 1 ml of medium (x 10^{10}). Viability was calculated by dividing living cells by dead cells and multiplying by a factor of 100%. The number of cells in 1 ml of solution was calculated by multiplying three factors

Table 1. Cell viability**(in % and 1 ml × 10^{10}) before/after treatment by low voltage electric current and Students *t*-test.

Groups	Control		Treatment		P-values	
	%	$\times 10^{10}$	%	$\times 10^{10}$	%	$\times 10^{10}$
1 Hz	63.31 ± 4.23	4.08 ± 1.80	79.17 ± 5.51	7.74 ± 0.45	0.016*	0.027*
4 Hz	55.50 ± 5,07	3.27 ± 1,29	69.87 ± 6,77	3.83 ± 0,80	0.042*	0.553
17 Hz	54.13 ± 5.08	3.52 ± 2.0	65.97 ± 3.05	3.85 ± 0.54	0.026*	0.795

* signifficance at level 0.05
** all test parameters were repeated 30x

(number of living cells, number of counted quadrants (5) and the dilution factor (10 000)) and dividing the result with the counting chamber volume (0.0001 ml).

Statistical analysis

The data gathered from viable samples was statistically processed using statistical analysis tests such as Student's t-test and single factor ANOVA. Main results were expressed as average values with standard deviations.

Results

Low voltage electric current treatment

According to Table 1, we have the average data (% and × 10^{10}) along with a standard deviation (± SD) for the control and treated group (1.4 and 17 Hz) of samples. Treating the samples with a low voltage electric current of different frequencies caused an increase of viability and overall cell number in all three of the used frequencies. The highest levels of increase in cell viability and cell number, was in the 1 Hz treatment (15.86% increase in viability and 3.66 $\times 10^{10}$ increase in cell number). The other two frequencies (4 and 17 Hz) had a slight increase in cell vi +ability and cell number.

As it could be seen in Figure 1, the differentiation between living and dead cells showed an increase in cells, which were metabolizing the dye at the time of the counting (light blue or dark grey color). These cells were not taken into account as we could not determine if they were dying or actively metabolizing the dye. So only colorless cells were counted as the living and the dark blue/dark grey counted as the dead.

Laser irradiation treatment

Treating the samples with laser irradiation caused an increase in both cell viability and cell number in both experimental groups (Table 2). Although, the highest cell viability increase (5.68%) was in green laser domain (532 nm), the highest cell number increase (3.39 × 10^{10}) was in red laser domain (648 nm).

Combined treatment

As presented in Table 3, the highest increase in cell viability had treatments of 1 Hz frequency and red laser beam (19.5%) and 17 Hz frequency and red laser beam as well (13.16%), while the lowest increase had the combination of 4 Hz frequency and red laser beam (5.5%). Cell number had highest values in 1 Hz frequency and green beam laser (8.32 × 10^{10}), 1 Hz and red beam laser (7.7 × 10^{10}), and 4 Hz frequency and green laser beam (7.45 × 10^{10}), while the lowest cell number had the 4 Hz frequency and red laser beam (2.2%).

Discussion

Past studies analyzing the effects of low voltage electric current on yeast are not abundant, although fusing of fungal

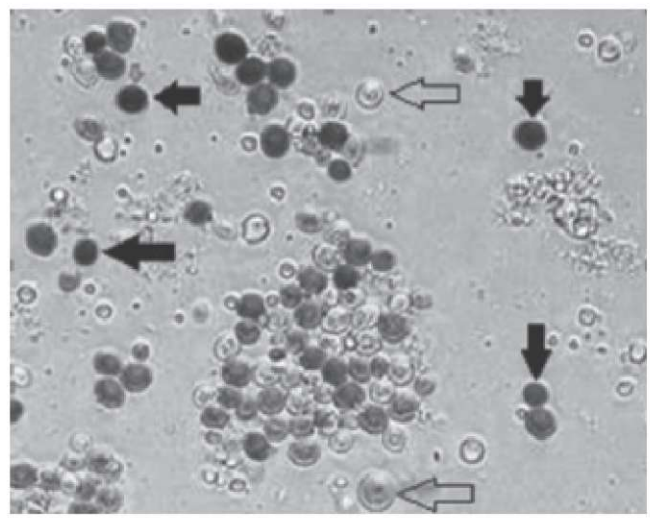

Figure 1. Methylene blue stain (black/white filter) was used to visualize alive (light arrow) and dead cells (dark arrow).

Table 2. Cell viability**(in % and 1 ml × 10^{10}) before/after treatment by laser irradiation and Students *t*- test.

Groups	Control		Treatment		P-values	
	%	$\times 10^{10}$	%	$\times 10^{10}$	%	$\times 10^{10}$
648 nm	67.54 ± 2.84	7.17 ± 0.45	71.82 ± 1.37	10.56 ± 3.26	0.079*	0.148
532 nm	69.49 ± 2.62	7.97 ± 1.29	75.17 ± 0.98	11.02 ± 1.03	0.024*	0.033*

* significance at level 0.05
**All test parameters were repeated 30x

Table 3. Cell viability**(in % and 1 ml × 10^{10}) before/after combined treatment (RL = red laser beam at 648 nm; GL = green laser beam at 532 nm) with single factor ANOVA values.

Groups	Control		Control +		Treatment		P-values	
	%	$\times 10^{10}$	%	$\times 10^{10}$	%	$\times 10^{10}$	%	$\times 10^{10}$
1 Hz+RL	55.90 ± 3.75	4.10 ± 0.33	71.50 ± 1.25	9.07 ± 2.0	75.40 ± 0.51	11.80 ± 1.75	0.000*	0.002*
1 Hz+GL	70.08 ± 0.50	7.15 ± 0.92	72.53 ± 1.40	10.87 ± 0.49	76.49 ± 2.70	15.47 ± 1.30	0.012*	0.000*
4 Hz+RL	66.47 ± 2.28	6.72 ± 0.41	70.50 ± 0.65	7.13 ± 0.39	72.97 ± 1.60	8.92 ± 0.38	0.008*	0.001*
4 Hz+GL	66.78 ± 3.34	7.35 ± 0.82	68.12 ± 0.34	8.37 ± 0.33	74.31 ± 0.85	14.80 ± 0.79	0.007*	0.000*
17 Hz+RL	58.90 ± 2.25	7.18 ± 0.38	67.84 ± 3.19	7.53 ± 0.42	72.06 ± 1.44	9.45 ± 0.48	0.003*	0.001*
17 Hz+GL	65.34 ± 1.74	6.43 ± 0.16	67.48 ± 1.34	6.57 ± 0.57	73.32 ± 2.36	8.68 ± 1.88	0.003*	0.003*

* significance at level 0.05
**All test parameters were repeated 30x

cell protoplasts under high voltage pulses was observed in earlier works (Somogyvári et al. 1996). Most of the related studies (Chen et al. 1998; Rodriguez et al. 2003; Schuerger et al. 2006) were focused on the duration of treatment and the voltage. Increasing of the voltage output leads to a decrease in numbers of living cells and in doing so affects actual viability by physically destroying yeast cell walls (if the voltage threshold exceeds 80 V). Extending the duration of treatment can also lead to decreases in viability and cell unit counts, as it prolongs the life cycle of fewer yeast generations. However, several authors (Binninger and Ungvichian 1997; Takeshita et al. 2003) propose the possibility of applicative characteristics of alternative technologies in the industrial sector. During the past few years, low voltage electric current with 1 mA charge was used in research in industrial-level production, however, this creates a higher charge within the bioreactor and can potentially burn down cells. Placing more strain on cells will most likely yield yeast cells with thicker cell walls and prolong cell division, which could produce abnormal cells.

When the samples were treated only with the laser irradiation protocol (either 648 or 532 nm beam) cell viability would rapidly rise for a longer time and would remain at a high induction level, in contrast to the low voltage electric treatment. According to several literature sources (Jacob et al. 1981; Chi et al. 1995; Wellman et al. 1996; Coster and Hillcot 1999; Takeshita et al. 2003), laser irradiation treatment leads yeast cells into a division-ready state as this speeds up their molecular-biological clock. The mechanism of inducing yeast cells to divide (even the cells physiologically not yet able to do) is still not fully understood. According to several authors (Petin et al. 2001; Schuerger et al. 2006; Bairva and Bastila 2010), excitation of specific cyclin-dependent kinases is responsible for activation/control/repression of the cellular life cycle.

According to Somogyvári et al. (1996), electric current pulses (optimally at 200 kHz and at 1 MHz best pearl chain formation) caused better protoplast fusion in *Mucor circinelloides*. This finding supports the fact that high frequencies and high voltage increase the success of fusion of protoplasts, however, this could lyse the walls of other organisms such as baker's yeast.

Based on aforementioned arguments, the results gathered in this research show a far greater increase in cell viability and cell growth under laser irradiation than under low voltage electric current at all three tested frequencies. One of the drawbacks of laser irradiation treatment was the high concentration of energy, which increases cellular temperature and literally fries yeast cells if not carefully calibrated for dosage.

Highly vacuole saturated yeast cells were noticed during treatments with both laser irradiation and electric current, although in much higher percentage in electric current treatments. This type of cells slow down cell growth by competing for more nutrients, while slowly dividing or not dividing at all, thus impairing their cell cycle mechanisms and becoming a colonial burden (Karba et al. 1991; Omori et al. 1996;

Nakasono et al. 1997; Stewart et al. 1999). Treatment series performed with the 532 nm laser indicated an increase in young yeast cells with an average of two newly formed buds on many cells. This situation leads to increased viability rates and a greater number of cell units in samples. For future research we recommend a detailed analysis of buds. Their presence/absence, as well as their size and age, could be a valuable indicative and prediction tool.

Combined treatments with low voltage electric current and either red or green beam laser have shown higher cell viability values and cell unit counts than separate treatment of samples (with only electric current or laser irradiation). Although, high resulting values may be achieved by separate treatments, this increased result is impacted by the fact that these treatments take relatively less time than combined treatments. By combining first the low voltage electric current and then laser irradiation, we actually prepare yeast cells for the shock-plateau, and when we irradiate them we have prolonged their cell division cycle by adding a small amount of irradiation energy.

Aggregation of yeast cells in colonies often leads to increased mortality rates. The presence of competition for nutrients is easily observed by (microscopically) analyzing samples and seeing smaller cells along with abnormally large ones. This competition is present even in nutrient rich mediums. Nutrient competition is also very common in control series and series, which need a 24 h waiting period. The logical assumption would be that this competition is forcing yeast cells to grow unhindered, while at the same time reducing available nutrients and pH values.

The amount of laser irradiation must be experimentally determined prior to treatment, because irradiation may trigger apoptotic cell mechanisms, which destroy cells much faster than the actual treatment. For this reason, combined treatments were found to be the most suitable. In these treatments the samples blasted with laser irradiation must cross over the energy excitation threshold (this threshold is raised by prior treatment with low voltage electricity) to actually affect the cells.

Cell viability results gained after 1 and 4 Hz treatments show a major advance in low-to-none collateral damage treatments. If we add the 17 Hz frequency, we get another improved treatment for increasing cell viability and cell growth, however, its higher frequency nature presents some limitations. The 17 Hz frequency increases cell viability rapidly, but then it becomes static and starts to kill yeast cells at a faster rate.

The 4 Hz frequency has shown to induce the budding process in young yeast cells, which led to increased cell viability (budding intensity is of indicative nature!). Due to its higher pulse nature, this frequency had a positive effect on overall cell number.

By forming new aggregated colonies, many yeast cells start to die out due to over-consumption of nutrients and higher percentage of already dead cells in these colonies. This process leads to subsequent cell viability loss and lower cell unit counts as the dead cells become a burden to the colony. With microscopic analysis of fresh slides treated with 4 Hz frequency, we noticed more budding young cells as well as large storage vacuoles in cells, which was explained by multi-sporulative existence of a haploid nature (even though the cells were diploids there was a possibility of forced polyploidy, which meant a shorter life cycle). In contrast to the 1 Hz, the 4 Hz frequency is more suitable for industrial applications as it provides a sufficient viability increase factor and is cheaper to produce.

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ARTICLE

Comparative effect of L-asparagine and sodium nitrate in inducing L-asparaginase production by endophytic *Fusarium* sp.

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ABSTRACT This study investigated the effect of two nitrogen sources (L-asparagine and sodium nitrate) on L-asparaginase production by endophytic *Fusarium* sp. (R19). L-asparagine is the more expensive nitrogen source, while sodium nitrate is the cheaper alternative of them. The production of L-asparaginase was quantified via Nesslerization and optimum incubation period was determined at 5-day intervals for 20 days. Fungal biomass obtained from supplementation of these nitrogen sources were weighed and correlated to the L-asparaginase production via Pearson correlation. Results revealed that sodium nitrate was unfortunately, inferior to L-asparagine in inducing L-asparaginase production in isolate R19. Supplementation with 1.25% L-asparagine yielded more L-asparaginase (3.01 U/mL), while the addition of sodium nitrate produced significantly lower levels (0.65 U/mL). The optimum incubation period was 5 and 10 days with supplementation of sodium nitrate and L-asparagine, respectively. Production of L-asparaginase correlates strongly to the fungal biomass (r = 0.990) suggesting nitrogen source impacts fungal growth and biomass, which subsequently influenced L-asparaginase production. To summarize, production of L-asparaginase from R19 was optimum with supplementation of 1.25% L-asparagine, incubated for 10 days. Sodium nitrate, although it is relatively cheap, was not effective in inducing L-asparaginase production. Further optimization studies can be performed to produce more L-asparaginase. **Acta Biol Szeged 60(2):145-150 (2016)**

KEY WORDS

anticancer
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endophytic
Fusarium
sodium nitrate

Introduction

L-asparaginase is an enzyme that catalyzes the conversion of non-essential amino acid L-asparagine to L-aspartic acid and ammonia. L-asparaginase can be used as a chemotherapeutic agent to treat acute lymphoblastic leukemia in children (Kenari et al. 2011). The conversion of L-asparagine in the body effectively deprives the cancer cells of the nutrients required to sustain their rapid growth (Lubkowski et al. 1996). Healthy normal cells are not affected as they have asparagine synthetase, which converts aspartic acid to L-asparagine (Theantana et al. 2007). L-asparaginase is produced by a wide range of organisms, primarily by bacteria, followed by various species of actinomycetes, fungi, algae and plants (Sarquis et al. 2004; Verma et al. 2007). Most of the commercially available L-asparaginase are produced from *Escherichia coli* and *Erwinia chrysanthemi* (Zalewska-Szewczyk et al. 2009).

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Over the years, it was discovered that therapeutic responses from bacteria-derived L-asparaginase has toxic side effects and causes immunogenic reactions (Capizzi and Cheng 1981; Baskar and Renganathan 2009). These reactions prompted investigations on the use of L-asparaginase from alternative non-bacteria sources. In this context, filamentous fungi are exploited as their eukaryotic nature shares closer phylogenetic relatedness with humans. It is hypothesized that due to the relatedness, hypersensitivity reactions are less likely to occur from fungal-derived asparaginase (Baldauf and Palmer 1993). Several filamentous fungi have been discovered to produce L-asparaginase, which include *Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp. (Sarquis et al. 2004). These fungal isolates are also prominent source of other secondary bioactive metabolites and enzymes with medical and industrial importance, such as amylases, cellulases and chitinases.

In this study, endophytes from medicinal plants are sourced and investigated for their production of L-asparaginase. Although endophytes can be found in almost all plants, medicinal plants have been identified as a favourable diverse repository of endophytic fungi with novel metabolites (Patil et al. 2012; Chow and Ting 2015). It is therefore the interest of

this study to validate this hypothesis. It is also the aim of this study to evaluate the response of endophytes towards nutrient substrates, in terms of L-asparaginase production. Nutrient substrates are generally known to have significant effect on the production of enzymes by various fungi. For *Aspergillus tamari*, high levels of L-asparaginase were produced when supplemented with 0.2% urea and 1% glutamine (nitrogen sources) (Sarquis et al. 2004). For *A. terreus* MTCC 1782, high levels were achieved with the supplementation of 2% proline, 1% sodium nitrate and 1% L-asparagine (Baskar and Renganathan 2009). Nevertheless, in some isolates, nutrient sources may repress the production of L-asparaginase, such as the case for *A. nidulans* where L-asparaginase production was lower in the presence of high concentrations of ammonium (Shaffer et al. 1988). Therefore, it is crucial that nutrient sources are investigated for their influence on L-asparaginase production.

In this study, the production of L-asparaginase by an endophytic fungal isolate (*Fusarium* sp., R19) obtained from *Andrographis paniculata* ("Hempedu Bumi"), is examined using two different nitrogen sources (sodium nitrate and L-asparagine). L-asparagine is more expensive than sodium nitrate, and the supplementation of L-asparagine may not be economically feasible on a large-scale basis. Both nitrogen sources have been used in other studies to obtain high L-asparaginase production due to their roles as essential nutrients for growth, for enzyme production, and synthesis of essential fats, proteins and carbohydrates (Ghasemi et al. 2008; Narayana et al. 2008; Baskar and Renganathan 2009). In addition to L-asparaginase production, biomass was also determined to understand the influence of nitrogen sources on endophyte growth (biomass) and the subsequent impact on L-asparaginase production. This study therefore reports our investigation on the influence of sodium nitrate and L-asparagine on the growth and production of L-asparaginase by endophytic *Fusarium* sp. (R19), and the feasibility of substituting L-asparagine with sodium nitrate for economical reasons.

Materials and Methods

Fungal culture establishment and identification

The fungal culture (R19) was previously isolated by Tan et al. (2013) from the roots of *A. paniculata* using surface sterilization methods. The fungal isolate was maintained on Potato Dextrose Agar (PDA; Difco™) at 25 ± 2 °C. Identification of the isolate was performed by first extracting the fungal genomic DNA using Vivantis GF-1 Nucleic Acid Extraction kit. This was followed by PCR using universal primers ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3'). Composition of PCR mixtures modified from White et al. (1990) was as follows: 5 µl of genomic DNA extract (~100 ng), 5-5 µl of primers (10-10 µmole), 25 µl of GoTaq @Green Master Mix 2x (Promega, Malaysia), and 10 µl of nuclease free water.

The reaction mixture was amplified in My Cycler Thermocycler (Bio-Rad). The process include initial pre-heating at 95 °C for 1 min, followed by 34 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 40 s, extension at 72 °C for 90 s and a final extension at 72 °C for 5 min. To confirm the success of the PCR reaction, the PCR products were subjected to electrophoresis using 1% (w/v) agarose gel supplemented with gel red (Biotium®), subsequently viewed with a UV transilluminator (Syngene®). The PCR products were then purified with the aid of Wizard® SV Gel and PCR Clean-up System (Promega), and outsourced to NHK BioScience for sequencing. The result was compared to the database from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and the accession number obtained.

Detection of L-asparaginase production

A qualitative plate assay was first conducted as described by Chow and Ting (2015) to verify the production of L-asparaginase by isolate R19. Based on their method, Czapek-Dox's agar supplemented with 3 mL of 2.5% phenol red (prepared in ethanol with pH adjusted to 6.2 using 0.1 mol/L NaOH) was first prepared and subsequently inoculated with mycelial plug (0.5 cm diameter) of R19. After 3 days of incubation, the production of L-asparaginase was detected by the formation of pink zone, with the diameter measured over a 7-day period. A quantitative assay to detect L-asparaginase production was also performed. For this, inoculation (5 mycelial plugs) was first performed in Czapek-Dox's broth and incubated for 5 days. The biomass was separated by filtration using Whatman No. 1 filter paper to obtain the filtrate containing the crude L-asparaginase. Enzymatic assay was performed on the crude enzyme obtained via Nesslerization to determine the L-asparaginase activity (Theantana et al. 2007). Nesslerization was performed in reaction tubes consisting of 200 µL of 0.04 M L-asparagine in 0.05 M Tris(hydroxymethyl) aminomethane (Tris-HCl) buffer (pH 7.2), 100 µL of 0.05 M Tris-HCl buffer (pH 7.2), 100 µL distilled water and 100 µL crude enzyme. The samples were incubated at 37 °C for 60 min prior to the addition of 1.5 M trichloroacetic acid (TCA) to stop the reaction. Then, 4.5 mL distilled water was added to the reaction tubes, followed by 400 µL of Nessler's reagent. The absorbance values of the samples were read at 450 nm and the enzymatic activity was expressed as the amount of ammonia formed (µL) per minute per mL enzyme used. The

assay was performed in triplicates. For controls, the procedure was repeated with the exception that the reaction was stopped using TCA prior to incubation.

Influence of nitrogen sources

To determine the influence of nitrogen sources, Czapek-Dox's broth was supplemented with L-asparagine (gradually substituted with sodium nitrate) in five different concentrations (0.25, 0.50, 0.75, 1.00, 1.25%, w/v). The concentration of the nitrogen source was designed based on the conventional optimization of nutritional requirement according to Chao and Lee (1994). The production of L-asparaginase was measured at a 5-day interval, from day 1 to 20, via Nesslerization as described in the earlier section, and expressed as L-asparaginase activities (U/mL). For the experiment, controls were prepared using fungal cultures cultivated without the supplementation of either nitrogen source (0% concentration). The biomass was weighed and these values were used in Pearson Correlation Test to determine the correlation between fungal biomass and L-asparaginase production.

Statistical analysis

The data was collected and One-way ANOVA was performed. Mean comparisons were analysed with Tukey test at $HSD_{(0.05)}$. The statistical analysis was performed using the SPSS software version 20. Pearson correlation test was performed to determine the interaction between fungal biomass and L-asparaginase activities.

Results

Identification and L-asparaginase production of Fusarium R19 isolate

Based on the sequence of the 18S rRNA gene, the fungal isolate R19 was identified as *Fusarium* sp. with the sequence deposited at Genbank and the accession number KT953312 assigned. This fungal isolate was positive for L-asparaginase production as formation of pink zone was detected from the plate assay. The diameter of the pink zone was 8.10 ± 0.12 cm after 5 days. The amount of the produced L-asparaginase was 2.53 ± 0.71 U/mL.

Influence of nitrogen sources

Of the two nitrogen sources, L-asparagine was more effective in inducing higher production of L-asparaginase in isolate R19 compared to sodium nitrate, with mean of L-asparaginase

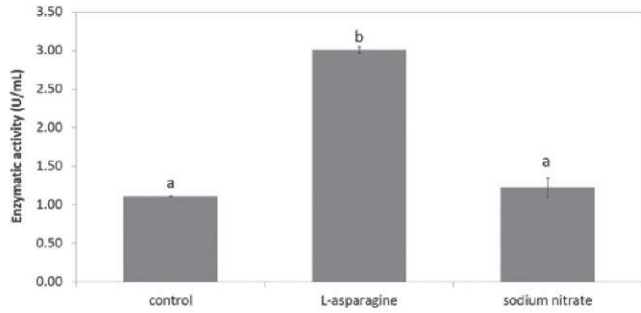

Figure 1. L-asparaginase production in response to nitrogen sources (L-asparagine, sodium nitrate) as compared to control. Bars indicate standard error of means. Means with the same letters are not significantly different according to Tukey grouping ($HSD_{(0.05)}$).

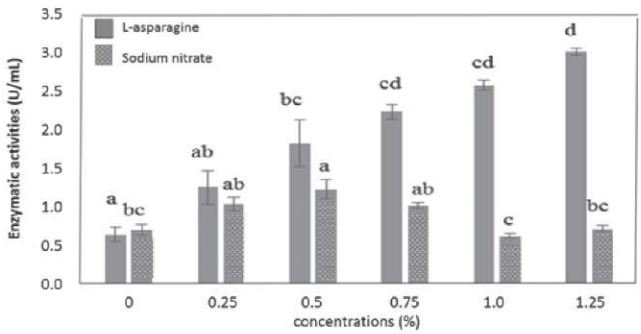

Figure 2. L-asparaginase production by endophytic *Fusarium* sp. (R19) under the influence of various concentrations of L-asparagine and sodium nitrate concentrations. Bars indicate standard error of means. Means with the same letters within nitrogen source are not significantly different according to Tukey grouping ($HSD_{(0.05)}$).

produced at 3.00 U/mL for supplementation with L-asparagine compared to 1.22 U/mL with supplementation of sodium nitrate (Fig. 1). Sodium nitrate did not stimulate production of L-asparagine as the amount was not significantly higher than levels detected in control (1.11 U/mL) ($p<0.05$) (Fig. 1). Isolate R19 responded favourably to increasing concentrations of L-asparagine, where 1.25 U/mL L-asparaginase was detected at 0.25% L-asparagine, doubling to 3.01 U/mL in 1.25% L-asparagine (Fig. 2). This suggested that isolate R19 was adaptable to supplementation of 1.25% L-asparagine. On the contrary, optimum production of L-asparaginase was achieved at 0.5% sodium nitrate, and further increase in concentrations of sodium nitrate (0.75, 1.0, 1.25%) resulted in a decrease in L-asparaginase production.

The optimum incubation period differed in response to the N sources. Optimum incubation was at day 10 with supplementation of L-asparagine (Fig. 3). On the contrary, the optimum incubation period with sodium nitrate supplementa-

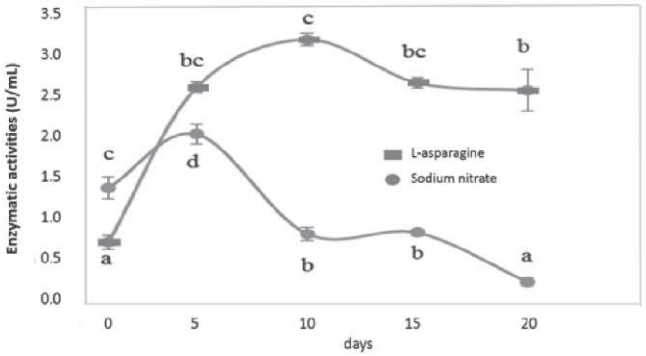

Figure 3. L-asparaginase production by *Fusarium* sp. (R19) observed throughout the 20-day incubation period, in response to L-asparagine and sodium nitrate supplementation. Bars indicate standard error of means. Means with the same letters are not significantly different according to Tukey grouping ($HSD_{(0.05)}$).

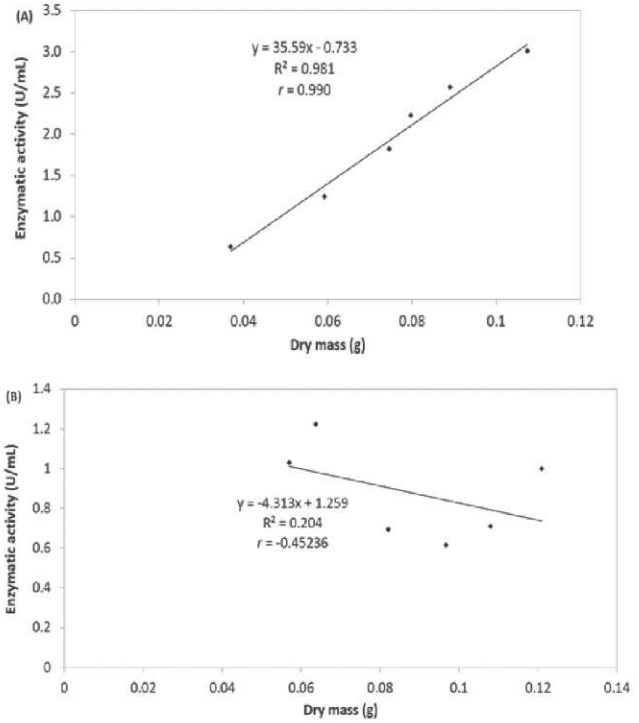

Figure 4. Correlation between L-asparaginase production and biomass of *Fusarium* sp. R19 in response to supplementation with (A) L-asparagine and (B) sodium nitrate.

tion was achieved earlier at day 5. A gradual decline in L-asparaginase production was observed thereafter, from day 10, 15, and 20 in both cases. The relationship between the fungal biomass and L-asparaginase production was established using the Pearson correlation test. A strong positive correlation was found between L-asparaginase production with fungal biomass supplemented with L-asparagine (r=0.9900) (Fig. 4A). This suggested that supplementation with L-asparagine increased the biomass, which also led to higher levels of L-asparaginase produced. On the contrary, sodium nitrate had an inverse correlation with biomass (r=-0.4523) (Fig. 4B). This suggested that there is poor/weak correlation between biomass and L-asparaginase production when supplemented with sodium nitrate. Sodium nitrate may promote growth but its presence also repressed L-asparaginase production. This result suggests that rather than the biomass, L-asparaginase production was more dependent on the nitrogen source. From Figure 4A and 4B, it can be concluded that the concentration of nitrogen sources affect the fungal growth to some extent.

Discussion

This study has revealed that endophytic *Fusarium* sp. from *A. paniculata*, shared similar L-asparaginase-producing traits as other non-endophytic *Fusarium* sp. (Hosamani and Kaliwalm 2011; Tippani and Sivadevuni 2012; Chow and Ting 2015). The L-asparaginase production was first detected by the formation of the pink zone, which was a result in an increase in pH (acid to alkaline pH) due to the accumulation of ammonia caused by L-asparaginase production (Chow and Ting 2015). The activity of L-asparaginase produced by *Fusarium* sp. (R19) was comparable to the activities of other L-asparaginase-producing endophytic fungi and *Fusarium* sp. For example, L-asparaginase activities by R19 were higher than endophytic *Colletotrichum* sp. and *Penicillium* sp. from Thai medicinal plants (0.014 to 1.530 U/mL) (Theantana et al. 2007), as well as when compared to *Fusarium solani* (0.32 to 2.21 U/mL) (Nakahama et al. 1973). Nevertheless, some species of *Fusarium* such as *F. equisetti* have far greater L-asparaginase activities (3.18 to 8.51 U/mL) (Hosamani and Kaliwalm 2011).

The stimulation of L-asparaginase production in media supplemented with L-asparagine may be due to L-asparagine being the suitable substrate of L-asparaginase therefore, creating a positive feedback mechanism leading to high production of L-asparaginase in L-asparagine supplemented media. For some isolates such as *Aspergillus terreus* MTCC 1782, addition of 1% L-asparagine into media gives maximum production of L-asparaginase (Baskar and Renganathan 2009). The positive response of *Fusarium* sp. R19 towards L-asparagine is similar to the response of *Aspergillus terreus* MTCC1782 towards L-asparagine, where L-asparaginase production increased until supplementation with 1.2% L-asparagine (Baskar and Renganathan 2011). This has also been observed in *Streptomyces albidoflavus*, where L-asparagine concentra-

tion over 1.0% decreased the production of L-asparaginase (Narayana et al. 2008).

The ineffectiveness of sodium nitrate contradicts with Baskar and Renganathan (2011). In their study, they found that groundnut oil cake, L-asparagine and sodium nitrate, were all proficient inducers of L-asparaginase in *A. terreus* MTCC1782. We postulate that the contradictory result may be attributed to the possible occurrence of nitrogen metabolite repression in the presence of nitrate for our isolate. It has been reported that with the presence of easily-assimilated nitrate, the synthesis of enzymes involved in secondary nitrogen source degrading pathway, such as L-asparaginase is repressed (Lee et al. 2011).

Harder and Dijkjuizen (1983) argues that under nitrogen limitation, microorganism may down-regulate the production of enzymes as an adaptive response. Thus, to maintain the production level, it is suggested to replenish the substrate each 10 days. Besides substrates, oxygen is also a limiting factor to L-asparaginase production (Mukherjee et al. 2000). Maintaining the substrate in the growth media and application of aeration are the key factors to keep the L-asparaginase production at a constant level (Mukherjee et al. 2000).

The positive correlation of biomass and with L-asparaginase production is a common trend in enzyme production, where enzyme production under the influence of L-asparagine is parallel to growth or biomass (Narayana et al. 2008). The result is confirmed by several other studies, indicating that the availability of nutrients in the media supports the growth of the fungi (Paustian and Schnurer 1987; Suberkropp 1995). The poor correlation in the presence of sodium nitrate, supports the nitrogen repression phenomenon discussed earlier. To the best of our knowledge, this is one of the few studies similar to Sarquis et al. (2004), in which concentration of nitrogen sources, biomass and L-asparaginase activity are measured concurrently.

In conclusion, *Fusarium* sp. R19 from *A. paniculata* has the potential to produce L-asparaginase that is useful for pharmaceutical use. L-asparaginase production was superior with the supplementation of L-asparagine (1.25%) compared to sodium nitrate. L-asparagine enhanced both the growth of R19 as well as the production of L-asparaginase. The supplementation with L-asparagine to isolate R19 did not lead to nitrogen repression, thus the addition of L-asparagine at higher concentration may further enhance L-asparaginase production. The alternative source, sodium nitrate was not a comparable substitute of L-asparagine.

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ARTICLE

Culture media supplemented with inorganic salts improve the growth and viability of several bacterial strains

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ABSTRACT In order to improve growth and storage conditions for bacterial cultures, commonly used basic culture media, Luria-Bertani broth (LB) and glucose-yeast extract (GY) were tested along with their supplemented versions (LBS and GYS) containing a complex set of inorganic salts required for common physiological processes. The growth kinetics and viability of 15 representative strains were compared on LB/LBS or GY/GYS. Growth kinetics were examined during a 24 h period. Five out of 15 strains showed enhanced growth on LBS and GYS. Three strains showed very low viability (3 months or lower) both on the basic and salt-supplemented media. Six strains could be equally recovered after 6 or 12 months both from LB/LBS and from GY/GYS. Six of the tested 15 bacterial strains showed significantly better recovery rate on the inorganic-supplemented media LBS or GYS than on basic LB or GY. These results show that inorganic supplement of basic media may significantly improve the growth and viability of several bacterial strains.
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KEY WORDS
growth kinetics
inorganic supplements
storage conditions
viability

Introduction

Efficient cultivation and reliable storage of bacterial cultures is a basic prerequisite in several microbiology-related fields, including human and veterinary medicine, environmental, industrial and agricultural microbiology, as well as molecular biology and biotechnological applications. For these purposes, mostly simple laboratory media are used which are based on hydrolyzed proteins (*e.g.*, meat extracts), yeast or various plant extracts. The general feature of these widely-used media (*e.g.*, Luria-Bertani broth, potato dextrose agar and glucose/yeast extract media) is that they are rich in organic compounds, but lack or contain only trace amounts of inorganic nutrients. On the other hand, plant tissue culture media always contain a complex set of inorganics (macroelements and microelements) required for growth and metabolism (George et al. 2012).

During the storage of various *Allorhizobium vitis* (formerly *Agrobacterium vitis*) strains we have observed that they lost their viability after a few months at 4 °C on glucose/yeast-extract (GY) slant agar, even if it was supplemented with $CaCO_3$ to buffer acid formation from glucose. When this medium was supplemented with AB salts (Lichtenstein and Draper 1986) the viability of *A. vitis* strains has increased up to ten months or longer. Similarly, the published 3-ketolactose medium (Bernaerts and De Ley 1963) containing only 1.0% (w/v) lactose and 0.1% (w/v) yeast extract was only weakly appropriate for this biochemical assay for *A. vitis* unless supplemented with AB salts (*our unpublished observation*). These data suggested us that the commonly used laboratory media do not contain the appropriate amount of inorganic nutrients. In an early study, Tartof and Hobbs (1987) found that phosphate supplement significantly increased the cell yield, and thereby the plasmid yield of *Escherichia coli*. A more recent study has shown that certain mono- (Na, K), and bivalent (Ca, Mg) cations had positive effects on the growth and viability of *A. vitis*, although data have not been presented on their action (Tanaka et al. 2009). They may contribute to transport or other basic physiological processes, or they are components of essential vitamins and coenzymes.

The Luria-Bertani broth (LB) and glucose/yeast extract media (GY) are widely used for bacterial cultures. In this study we compared the effects of the original LB and GY, and their modified formulae supplemented with inorganic salts on the growth and storability of various bacterial cultures. Our results show that inorganic supplements may significantly increase the viability of certain strains.

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Table 1. Bacterial strains.

Code*	Strain	Reference**	Growth conditions (medium/ temperature)
SZMC 21395	*Agrobacterium tumefaciens* C58 (= *Agrobacterium fabrum*)	Sciaky et al. 1978	GY/25 °C
SZMC 21396	*Allorhizobium vitis* Tm4 (= *Agrobacterium vitis*)	Szegedi et al. 1988	GY/25 °C
SZMC 21397	*Allorhizobium vitis* AT1	Szegedi et al. 1988	GY/25 °C
SZMC 21398	*Allorhizobium vitis* S4	Szegedi et al. 1988	GY/25 °C
SZMC 0209	*Bacillus subtilis*	n.a.	LB/30 °C
SZMC 21399	*Escherichia coli* DH5α	Hanahan et al. 1991	LB/37 °C
SZMC 21400	*Escherichia coli* LE392	Zyskind and Bernstein 1992	LB/37 °C
SZMC 21401	*Burkholderia phytofirmans* PsJN	Toklikishvili et al. 2010	GY/25 °C
SZMC 6269	*Chromobacterium violaceum* wt85	n.a.	LB/25 °C
SZMC 21402	*Erwinia amylovora* K21	Hevesi, M., personal communication	GY/25 °C
SZMC 21403	*Novosphingobium* sp. Rr 2-17	Gan et al. 2012	GY/25 °C
SZMC 21404	*Pseudomonas fluorescens*	n.a.	LB/25 °C
SZMC 0567	*Serratia marcescens*	n.a.	LB/30 °C
SZMC 21405	*Serratia plymuthica* IC1270	Dandurishvili et al. 2010	LB/30 °C
SZMC 0227	*Streptococcus faecalis*	n.a.	LB/30 °C

*SZMC: Szeged Microbiology Collection, Szeged, Hungary, **n.a.: not available

Materials and Methods

Bacterial strains

Strains used in this work (Table 1) were stored in glycerol stocks at -80 °C. For daily use they were cultured on GY or LB media at 25, 30 or 37 °C (Table 1) for two days, and then stored at 4 °C.

Media

Culture media were based on LB or GY (Table 2) and prepared in deionized water. The $CaCl_2 \times 2\ H_2O$ stock solution (3% calcium-chloride, w/v) was autoclaved in aliquots and stored at 4 °C. For FeEDTA stock solution, 1.12 g $FeSO_4 \times 7\ H_2O$ and 1.5 g Na_2EDTA were completely dissolved separately in 100-100 ml water, then mixed and stored in aliquots at -20 °C. The microelement stock solution contained 1.0 g $MnSO_4 \times 7\ H_2O$, 0.5 g $ZnSO_4 \times 7\ H_2O$, 25 mg $Na_2MoO_4 \times 2\ H_2O$ and 2.5 mg $CoCl_2 \times 6\ H_2O$ dissolved separately, mixed and made up to 100 ml. Aliquots were kept at -20 °C. All solutions were prepared in deionized water. Since both tryptone and yeast extract contain the sufficient amount of amino acids, nitrogen-source (ammonium or nitrate salt) was not added. Media supplemented with the inorganics (Table 2) were designated as LBS or GYS. The pH was adjusted to 7.0-7.2 with 3% NaOH when it was necessary. Solid media were prepared with 1.2% (w/v) agar. Bacterial suspensions were made in phosphate-buffered saline (PBS), containing 1.236 g/l Na_2HPO_4, 0.18 g/l NaH_2PO_4 and 8.5 g/l NaCl.

Growth on agar plates

To get preliminary data about the suitability of LB and GY for a given bacterium, the growth of the tested strains were compared on solid GY and LB media as well as on their salt-supplemented derivatives LBS and GYS (Table 2) prepared with 1.2% (w/v) agar. Plates were inoculated with a loopful bacterial suspension (OD_{600} = 0.2 for the two *E. coli* strains and OD_{600} = 0.8 for the others) and then incubated at 25, 30 or 37 °C depending on the strain (Table 1). Bacterial growth was recorded after 24 and 48 hrs.

Table 2. Culture media used for bacterial growth and storage studies.

Medium	Components	Concentration
Luria-Broth medium (LB)	triptone	10.0 g/l
	yeast extract	5.0 g/l
	NaCl	5.0 g/l
Glucose-yeast extract medium (GY)	glucose	10.0 g/l
	yeast extract	5.0 g/l
	$CaCO_3$ (for storage only)	2.0 g/l
Inorganic supplement medium	K_2HPO_4	2.0 g/l
	KH_2PO_4	1.0 g/l
	$MgSO_4 \times 7\ H_2O$	0.5 g/l
	$CaCl_2 \times 2\ H_2O$ stock*	10.0 ml/l
	FeEDTA stock*	5.0 ml/l
	Microelement stock*	1.0 ml/l

*see Materials and Methods

Table 3. Viability of tested microorganisms on different storage media at 4 °C.

Code	Strain	Medium*	3	6	9 months	12	Viability months
SZMC 21395	*Agrobacterium tumefaciens* C58	GY	+	+	+	+	12
		GYS	+	+	+	+	12
SZMC 21396	*Allorhizobium vitis* Tm4	GY	+	+	+	(+)	12
		GYS	+	+	+	+	12
SZMC 21397	*Allorhizobium vitis* AT1	GY	+	(+)	-	n.t.	6
		GYS	+	+	+	+	12
SZMC 21398	*Allorhizobium vitis* S4	GY	+	(+)	-	n.t.	6
		GYS	+	+	+	+	12
SZMC 0209	*Bacillus subtilis*	LB	+	+	+	+	12
		LBS	+	+	+	+	12
SZMC 21399	*Escherichia coli* DH5α	LB	+	(+)	-	n.t.	6
		LBS	+	+	(+)	-	9
SZMC 21400	*Escherichia coli* LE392	LB	+	(+)	-	n.t.	6
		LBS	+	+	(+)	-	9
SZMC 21401	*Burkholderia phytofirmans* PsJN	GY	+	(+)	-	n.t.	6
		GYS	+	+	+	+	12
SZMC 6269	*Chromobacterium violaceum* wt85	LB	-	n.t.	n.t.	n.t.	0
		LBS	-	n.t.	n.t.	n.t.	0
SZMC 21402	*Erwinia amylovora* K21	GY	+	(+)	-	n.t.	6
		GYS	+	+	+	+	12
SZMC 21403	*Novosphingobium* sp. Rr 2-17	GY	+	+	+	+	12
		GYS	+	+	+	+	12
SZMC 21404	*Pseudomonas fluorescens*	LB	+	-	n.t.	n.t.	3
		LBS	+	-	n.t.	n.t.	3
SZMC 0567	*Serratia marcescens*	LB	+	-	n.t.	n.t.	3
		LBS	+	-	n.t.	n.t.	3
SZMC 21405	*Serratia plymuthica* IC1270	LB	+	+	-	n.t.	6
		LBS	+	+	-	n.t.	6
SZMC 0227	*Streptococcus faecalis*	LB	+	(+)	-	n.t.	6
		LBS	+	+	-	n.t.	6

*see Materials and methods. Strains were recovered on LBS (*Pseudomonas fluorescens*, *Serratia plymuthica* IC1270, *Escherichia coli* LE 392 and *E. coli* DH5α), or on GYS (other strains).

Growth kinetics in liquid media

Strains were cultured for 24 h in 48-well microtiter plates using the basic media (LB and GY) and media supplemented with inorganics (LBS or GYS). All strains were precultured in LB or GY liquid media with shaking for 24 h at appropriate temperatures. Then 1 ml aliquots of cultures were centrifuged and the pelleted cells were washed three times with 0.2% glycerol. These stocks were used during the subsequent experiments. Each well contained a cell concentration of 10^5 cells/ml. The OD_{600} value was measured in every hour during the 24 h growth period with a SPECTROstar Nano microplate reader (BMG Labtech, Ortenberg, Germany).

Storage experiments

Strains were inoculated parallely in three replicates onto LB and LBS slant agar media, or GY and GYS slant agar media supplemented with 0.2% $CaCO_3$ (Table 3) as it was found appropriate for a particular strain. Then cultures were incubated for 2 days at 37 °C (for *E. coli* LE 392 and DH5α) or at 25 or 30 °C (for the other strains, Table 1), and then stored at 4 °C. Viability of the strains was checked after three, six, nine and 12 months. To this end, loopful samples were suspended in 1.2 ml PBS and one loop was inoculated onto LBS (for strains kept on LB and LBS) or onto GYS (for strains kept on GY or GYS) plates. Recovery of strains was scored after three days of incubation at 37 °C (for *E. coli* LE 392 and DH5α) or at 25, or 30 °C (for the other strains).

Results

Growth of bacterial strains on solid media

During a preliminary survey, all strains were inoculated from suspensions onto LB and GY as well as their salt-supplement-

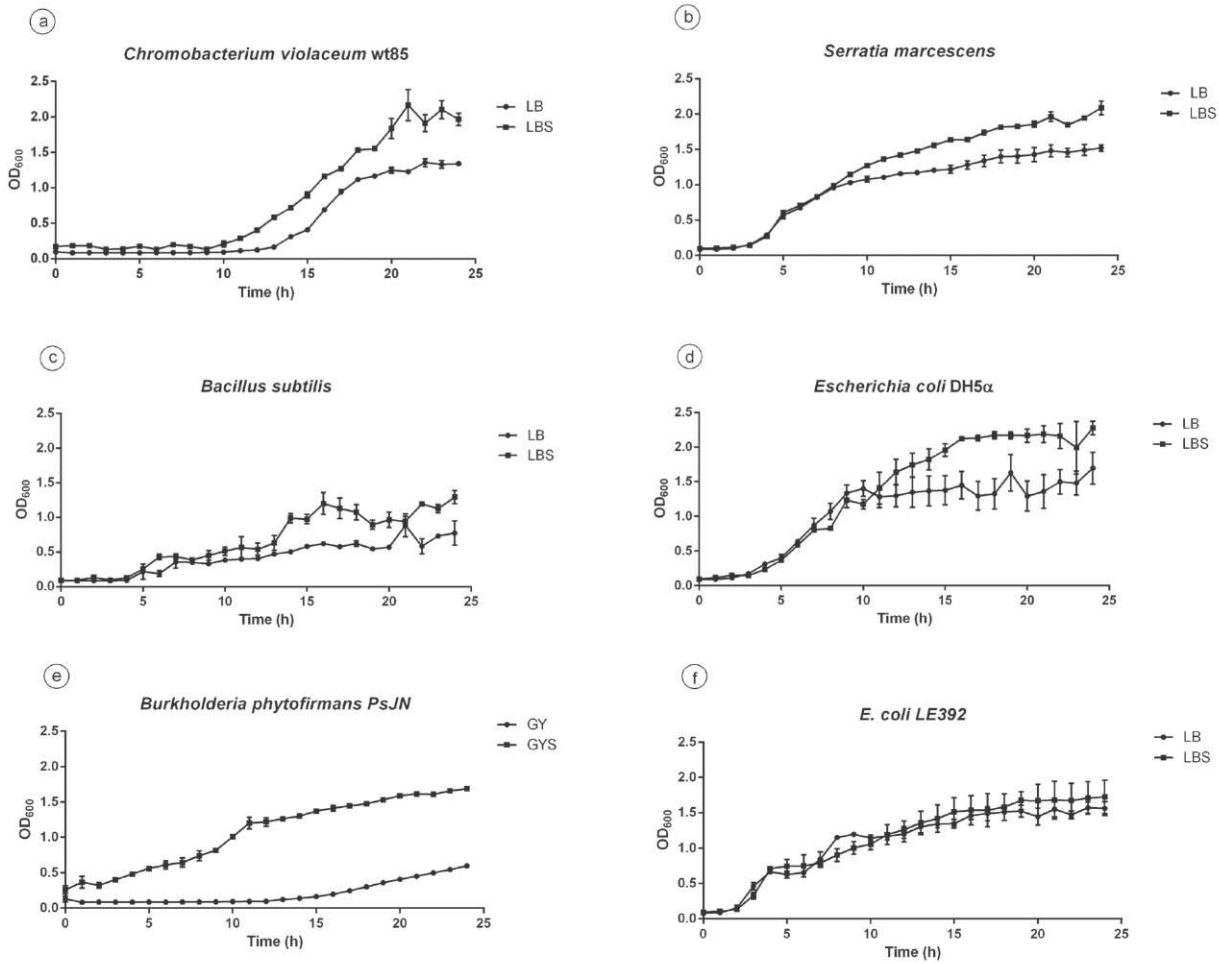

Figure 1. Growth kinetics of *C. violaceum* wt85 (a), *S. marcescens* (b), *B. subtilis* (c), *E. coli* DH5α (d), *Burkholderia* sp. PsJN (e), and *E. coli* LE392 (f) in liquid culture media. Some of these strains (a, b, c, d) showed more intensive growth in LBS medium than in LB, and one of them (e) showed more intensive growth in GYS medium than in GY. In case of *E. coli* LE392 (f) no growth difference was detected in the two tested media. Results are presented as mean ± SEM.

ed derivatives LBS and GYS in order to find an appropriate medium for each strain. After 24 h and 48 h growth, visual scorings of the plant associated bacteria *Agrobacterium tumefaciens* C58, *Allorhizobium* spp., *Burkholderia* sp., *Erwinia* sp. and *Novosphingobium* sp. showed better growth on GY-based media than on LB. The other strains preferred LB-based media to GY. Thus, for storage studies the appropriate media were prepared based on these observations. After 24 h of growth, *A. vitis* strains Tm4, AT1 and S4 as well as *E. amylovora* K21 and *Bacillus subtilis* showed more intensive growth on GYS than on GY medium. The two *E. coli* strains LE9392 and DH5α also grew better on LBS than on LB. These differences in growth on the basic and salt-supplemented media were still clear after two days for the three *A. vitis* and two *E. coli* strains. The other bacteria did not exhibit seemingly higher growth rates (data not shown).

Growth kinetics of bacterial strains in liquid media

The investigations made in liquid media were performed after the preliminary selection of the most appropriate medium for each strain (see above). *Chromobacterium violaceum, Serratia marcescens, B. subtilis* and *E. coli* DH5 showed more intensive growth in LBS media than in LB (Fig. 1 a, b, c, d). In the case of *E. coli* LE392 we did not detect any differences in growth (Fig. 1f). *Burkholderia* sp. PsJN grew better in GYS than in GY (Fig. 1e). The other strains did not show significant differences. We compared the results obtained on solid and in liquid media. The growth of *C. violaceum, S. marcescens, B. subtilis* and *E. coli* DH5α were enhanced on both solid and liquid media in the presence of inorganic elements. In the case of *E. coli* LE392, growth enhancement was

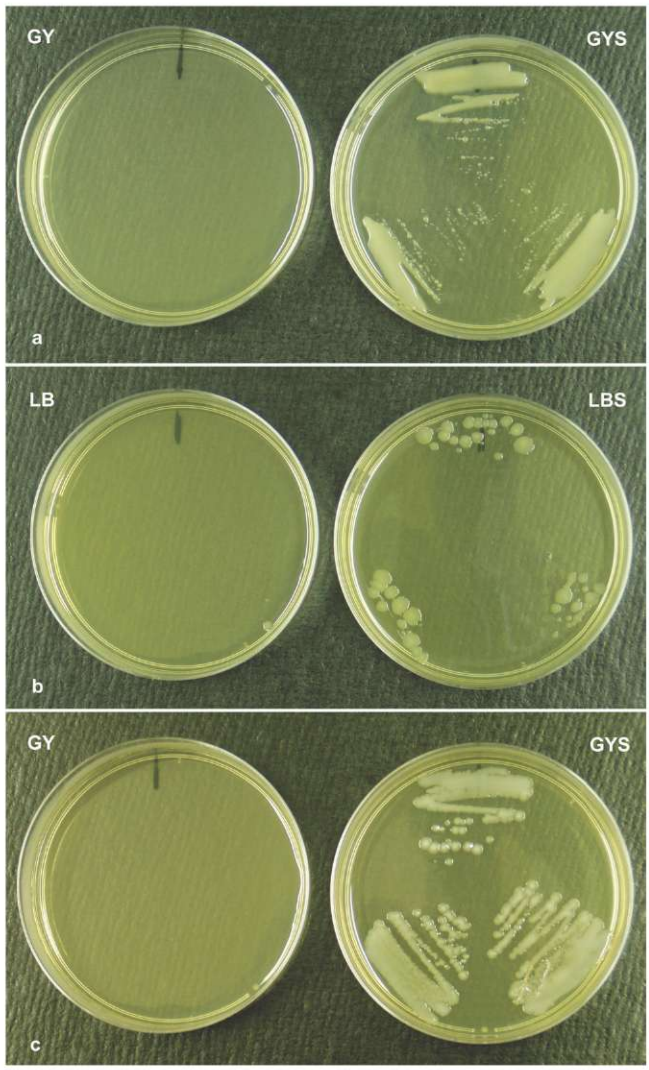

Figure 2. Recovery of *A. vitis* AT1 (a), *E. coli* LE392 (b) and *E. amylovora* K21 (c) strains on basic GY or LB (left) and salt-supplemented GYS or LBS media (right) after nine months of incubation at 4 °C. Each pair of plates shows the results of three replicates.

observed only on solid media. We observed the same results for the other tested strains; enhancement was achieved only on solid media (data not shown).

Viability of strains on basic and supplemented media

The tested strains highly varied in their viability during storage at 4 °C on basic LB/LBS or GY/GYS media solidified with agar (Table 3). The first group showed a very weak recovery or complete loss of viability after three to six months of storage in active cultures regardless of the media (LB and LBS) compared. The second group including six of the tested

Table 4. Summarized data on the viability of the tested bacterial strains stored at 4 °C on basic and inorganic-supplemented media.

Group I. Strains with low viability (≤3 months) on both media*	Group II. Strains with equal viability (6-12 months) on both media*	Group III. Strains with better viability on LBS or GYS medium than on the basic medium*
C. violaceum wt85	*A. tumefaciens* C58	*A. vitis* AT1
P. fluorescens	*A. vitis* Tm4	*A. vitis* S4
S. marcescens	*B. subtilis*	*E. coli* DH5α
	Novosphingobium sp. Rr2-17	*E. coli* LE392
	S. plymuthica IC1270	*B. phytofirmans* PsJN
	S. faecalis	*E. amylovora* K21

*LB or LBS, and GY or GYS, see also Table 3.

15 strains remained viable up to 6-12 months, but differences in their recovery from the basic and salt-supplemented media were not observed. Six strains, namely *A. vitis* AT1 and S4, *E. coli* LE392 and DH5α as well as *E. amylovora* K21 and *Burkholderia phytofirmans* PsJN could be recovered only until six month of storage from the basal medium (LB or GY), but they remained viable up to 9-12 months on the salt-supplemented LBS or GYS media (Fig. 2). These data are summarized in Table 4.

Discussion

Basic bacterial media like LB and GY are usually rich in organic compounds, but they contain low amounts of basic inorganics. Therefore we supplemented these media with various anions and cations, which are components of nucleoside triphosphates, nucleic acids and enzymes or contribute to various physiological processes. Growth kinetics of five strains were enhanced after using supplementation with inorganics in both solid and liquid media. No difference was detected in liquid cultures in the case of *E. coli* LE392, although its growth was slightly enhanced on solid media. The inorganic supplement also improved the viability, thus increased the recovery rate of several bacteria (six of the 15 tested strains).

Our data confirm the previous observations on the salt requirements of *A. vitis* (Tanaka et al. 2009) and extend these observations to other bacterial genera. Thus, the storage of bacterial cultures in small laboratories, where ultra-freezer is not available may become much safer with less frequent culture transfer. It also reduces the risks of contamination and failures due to missing strain designation. Considering these results, for daily laboratory work we propose the application of the inorganic-supplemented versions of the commonly used bacterial media, including LB, GY and others, containing

simple organic extracts. Our results may be adapted to other natural bacterial or fungal media (*e.g.*, PDA) to improve the efficiency of isolation from bacterial populations of various environmental, plant, food or medical samples.

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ARTICLE

Effects of 6-epi-ophiobolin A on mechanical and physiological parameters of cardiomyocytes and on the reorganization and amounts of their kv4.x ion channels

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ABSTRACT Ophiobolins are important members of the family of phytotoxic metabolites, and they possess antitumor, antibacterial, antifungal activities. At the same time, the knowledge on the effect of ophiobolins on ion channels is very limited. The voltage-dependent transient outward currents (I_{to}) are determined by kv4.2 and kv4.3 ion channels. Increasing evidence points to a role of HMGB1 and synapse-associated protein 97 (Sap97) as underlying factors related to inflammation. The Sap97 associates with kv4.x type channels in the complex and modulating their kinetic properties. Our hypothesis was that Sap97 mainly localizes in the intercalated discs of the cardiac muscle, therefore the altered expression of ion channels is presumably involved in the inhibition of normal mechanical parameters and physiological function of transient outward current (I_{to}). Our approach was to use purified 6-epi-ophiobolin A as effective molecular tools in the investigation of ion channels on cardiomyocytes. In this work, we have investigated the mechanical factors by atomic force microscope (AFM) technique and the surface expression of kv4 ion channels on the cardiomyocytes using confocal microscopy after immunofluorescence labeling. AFM study revealed that the elasticity of the cell surface, the Young's modulus had been moderately changed, as well as the cell volume and the heights of the cells in the presence of low concentration of 6-epi-ophiobolin A. After this treatment the Sap97 binding to kv4.3 or kv4.2 channels and distribution of their complexes are also changed in the membrane accompanied by altered physiological behavior of cardiomyocytes as compared to control. A growing number of researches using these new reductionist models of inflammation on cardiomyocytes, demonstrate a role of Sap97 in specific ion channel stability important for cardiac functions. These results suggest that Sap97 deactivation or reduction can lead (directly or indirectly) to changes in the functional cell surface expression of kv4.x channels with mechanical parameters, with biophysical and biochemical properties of cardiac I_{to} current.
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KEY WORDS

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Introduction

Nowadays, the effects of the secondary metabolites come into sight since they can influence the electrolyte balance in humans or animals, but their biological consequence is not well known. The Ca^{2+}-independent voltage-gated transient outward current (I_{to}) is important for maintenance of ionic currents in the first period of the repolarization phase of action potential in cardiomyocytes (Heufbach et al. 2003). Transient outward current is evolved mainly by kv4.2 and kv4.3 in human cardiomyocytes, and acts in the first period of repolarization phase of the action potential (AP) (Liu et al. 2015).

An essential characteristic that differentiates the voltage-gated potassium (Kv) channels from both the cardiac sodium (Nav) and calcium (Cav) channels is the variation in terms

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of electrophysiology and function of the K^+ channels (Nerbonne and Kass 2005). Two classes of repolarizing cardiac Kv currents can be mainly distinguished; the transient outward potassium current (I_{to}), which plays an important role in phase I of the AP, and the delayed, outwardly rectifying potassium current (I_K) (Wettwer et al. 1994; Nattel et al. 2007). At membrane potentials, above 30 mV both of these types are activated (Barry and Nerbonne 1996). Within these two major groups, there are further subdivisions of the I_{to} and I_K depending on various time- and voltage properties. Due to the different densities and biophysical qualities of these channels throughout the various regions of the heart, various waveforms of APs could be observed. For example, regional differential expressions of transient Kv currents were found between in the left ventricular endocardium and epicardium. The first region showed lower I_{to} density and very slow recovery, while in the second region, higher I_{to} density and fast recovery were observed (Brahmajothi et al. 1999). This variation of the distribution and kinetics of the I_{to} must be an important factor in the regulation of the physical cardiac rhythm and an even more significant factor in the pathological heart (Nerbonne and Kass 2005; Wettwer et al. 2013).

Furthermore, the physiologically active kv4.x channels are associated with several ancillary subunits such as β-subunits (kvβ1 and kvβ2), Kv channel-interacting proteins (KCHIP) acting as chaperons (Akar et al. 2004). KCHIPs regulate the surface expression of channels and determine the electrophysiological properties. The kv4.x ion channel function is dependent of the Kv channel complex specific subcellular localization and trafficking mechanisms (Kääb et al. 1998; Takeutchi et al. 2000; Chang-Liao et al. 2015). Kv4.x channel complex with kv4.3 pore-forming α-subunits, and auxiliary subunits KCHIPs, DPP6 and DPP10 were found as components of kv4.x channel complex modulating their currents in brain and in the myocytes as well as in other tissues (Radicke et al. 2005; Maffie and Rudy 2008; Cotella et al. 2010, 2012; Turnow et al. 2015). These genes with others for ion channel proteins are involved in the consequences of cardiomyopathy such as in the arrhythmogenesis of coronary artery disease and ventricular arrhythmias with sudden cardiac death (El-Haou et al. 2009; Nattel et al. 2007; Gaborit et al. 2009). Kv4.x α-subunits associate with synapse-associated protein 97 (Sap97) kinase (El-Haou et al. 2009; Fourie et al. 2014) and with high-mobility group box-1 (HMGB1) non-histone chromatin-associated protein, which modulates the normal physiological function of ion channels (Wang et al. 1999; Au et al. 2000; Yang et al. 2004, 2005; Liu et al. 2010; Kang et al. 2014). The Sap97 anchors the kv4.x ion channels and regulates the subcellular translocation and localization of the channels (Fourie et al. 2014). Kv4.x has been proven to concentrate in the sarcolemma of the ventricular myocytes at the intercalated disk region, which connects myocytes (Milstein et al. 2012; Liu et al. 2015).

HMGB1 performs diverse intracellular and extracellular functions in health and disease. In the nucleus, it regulates chromatin architecture, transcription, replication and repair processes. In addition, HMGB1 secreted by macrophages and necrotic cells also performs various intracellular and extracellular activities as an alarming danger signal during injury and diseases such as inflammation, arthritis, multiple human diseases including infectious diseases, ischemia, immune disorders, neurodegenerative diseases, metabolic disorders and cancer (Hock et al. 2007; Lei 2013; Kang et al. 2014, Roy et al. 2016). HMGB1 is the prototypic damage associated molecular pattern molecule (DAMP). HMGB1 is in conjunction with other factors, thus has cytokine, chemokine, and growth factor activity, orchestrating the inflammatory and immune response. HMGB1 location and function largely depend on redox states (Qiu et al. 2011).

The objective of Atomic Force Microscope (AFM) is the micro- and nanometer scale characterization of different biological samples in different fields such as peptides and oligonucleotides (Butt and Jaschke 1995; Bálint et al. 2007), photosynthetic reaction centers, membrane proteins (Bálint et al. 2007, Caloni et al. 2012) and the investigation of living cell's surface and their responses to different extracellular stimuli also available (Bálint et al. 2007; Wilhelm et al. 2007; Harmati et al. 2016). The AFM's possibility to work in liquid environment and especially the design of the MFP 3D type AFM made possible to study living cells *in situ*, in their physiological circumstances.

Most of the microbial secondary metabolites have substantial effects on humans and animals, but the background of their action is usually unclarified. The molecular mechanisms associated with these bioactive metabolites underlying stress conditions of I_{to} are still largely unknown.

Ophiobolins are a group of naturally occurring sesterterpene-type secondary metabolites with a specific tricyclic ring system (5-8-5) named as A-, B- and C-ring, respectively. They are mainly produced by filamentous fungi belonging to the genus *Bipolaris*, however, several ophiobolin analogues have been reported from other genera, such as *Drechslera*, *Cephalosporium*, *Ulocladium* and *Aspergillus* (Wei et al. 2004; Wang et al. 2013). Until now, 50 different ophiobolin analogues have been reported and characterized, 18 of them in the last five years. Interestingly, most of the ophiobolins were isolated from terrestrial microorganisms; however, many of the recently discovered ophiobolin analogues were isolated from marine microorganisms, which have become preferable sources of new bioactive compounds in the last decade (Wei et al. 2004; Wang et al. 2013; Bencsik et al. 2014). The best-known member of the ophiobolin family, the ophiobolin A exhibits a broad spectrum of biological activities such as phytotoxic, calmodulin antagonist, antimicrobial, antimalarial, nematocid or anticancer effects. Nowadays, the effect of ophiobolin A was demonstrated on big conductance

Ca-channels (BKCa): it decreased the BKCa channel activity (Bury et al. 2013).

In this study, we investigated whether the 6-epi-ophiobolin A (6EOPA) is able to modulate the distribution of kv4.x ion channels or there is any relation of the treatment to inflammation or has any effect to the mechanical characters of the cardiomyocytes. Furthermore, the molecular details about the association between the kv4.x channels and Sap97 subunits were also investigated. Our approach was to compare the localization and distribution of kv4.x channels and the anchoring protein Sap97 in the cardiomyopathy. For this reason, the localization and distribution of kv4.x channels and their modulators like Sap97 were recorded in the presence of derivatives of 6EOPA in cardiomyocytes under external stress caused by fungal metabolites. For the assays, the heart cell line of H9c2 rat was used as a tool to localize kv4.3 ion channels with correlated confocal microscopic methods with immunohistochemistry. Furthermore, understanding the cell model the AFM technique was applied to measure "stiffness" on the surface of cardiomyocytes showed the alteration of the cell surface, which could lead to change the pharmacological properties of ionic currents in cardiac cells.

Materials and Methods

Cell cultures of rat cardiomyocytes and cell treatment

Rat cardiomyocyte H9c2 cell line was a gift from E. Duda (HAAS, BRC, Szeged, Hungary), which were grown in Dulbecco's modified Eagle's medium (PAA, Austria) supplemented with 10% foetal bovine serum (FBS; PAA, Austria), 100 U/ml penicillin and 100 mg/ml streptomycin (Pen-Strep; Lonza, Hungary) in standard conditions (37 °C, 5% CO_2). The cells were grown in round glass coverslip, until 70% density both for immunofluorescence and AFM studies. For the treatment, 6EOPA was used at 2.8 x 10^{-6} mg/ml final concentration added to the medium. After 24 h, the cells were attached to the coverslip and stored at -20 °C until use both for fluorescence immunocytochemistry and AFM investigations.

To test the cytokine effects, antibody of anti-HMGB1 was used (GST-HMGB1 61 ng/ml in the medium, it marked as HMGB1), where the concentration was considered according to the literature (Liu et al. 2010; Szénási et al. 2013).

Immunofluorescent study on cardiomyocytes

For the immunofluorescent staining, the cells were blocked with 2% BSA in calcium-free phosphate-buffered saline (PBS) and incubated for 2 h at room temperature either with rabbit anti-kv4.2 (diluted 1:100; Alomone, Israel) or anti-kv4.3 (diluted 1:100; Sigma-Aldrich, Hungary) and mouse anti-Sap97 antibody (diluted 1:300; LifeSpan BioSciences, USA). Cells were then washed and incubated with Alexa 488-labeled anti-rabbit IgG (diluted 1:400, Molecular Probes) plus cy3-conjugated anti-mouse IgG (diluted: 1:400; Jackson Immunoresearch, USA) for 1 h at room temperature. Nuclei were stained with 1 µg/ml Hoechst 33258 (Sigma-Aldrich, Hungary) in PBS for 10 min. After rinsing, sections were mounted with fluorescent mounting medium (Agilent, USA), viewed and photographed with a confocal laser scanning microscope using an Olympus Fluoview FV1000 (Olympus, Germany).

Purification of 6-epi-ophiobolin A

The purification and separation of 6EOPA was performed by semi-preparative HPLC method from the ferment broth of *Bipolaris oryze* (SZMC 13003) based on earlier report (Bencsik et al. 2014). The identification of the 6EOPA compound (purity >96%) was carried out using LC-MS analysis and NMR examinations.

Measuring nanomechanical parameters of cells by atomic force microscope technique

The surface morphology, topography, and mechanical properties of 6EOPA treated cells were evaluated by using an AFM coupled with an inverted optical microscope (Axiovert 200, Zeiss, Hungary). Briefly, the combined inverted optical stage helped precise lateral positioning of the AFM tip over the region of cell tissues of target materials. The measurements were carried out with an Asylum MFP-3D head and Molecular Force Probe 3D controller (Asylum Research, USA) in force mode. The driver program MFP-3D Xp (ver. 13.17.101) was written in IGOR Pro software (ver. 6.37, Wavemetrics, USA). The silicon nitride cantilevers (Bio-Lever BL-RC150VB-C1, Olympus, Japan) with the nominal spring constant of 30 pN/nm in air were used for the experiments came from the same wafer. Calibration of the real spring constant of each individual cantilever was performed by thermal fluctuation technique (Butt and Jaschke 1995; Proksch et al. 2004) and the Sader method (Sader et al. 2012). The scan size was adjusted to 80 × 80 µm^2 to accommodate typical surface features, while maintaining a high resolution. The Young´s modulus was subsequently determined by using the Hertz´s contact model. For calculation of material stiffness, the Poisson's ratio was assumed 0.5, the ramp size was 1 µm, and the loading speed was 1 µm/s. A series of indentation forces (0.5-10 nN) were tested to calibrate, whether the indentation depth was in the proper range between 20-200 nm to prevent substrate surface defects and Hertz´s model limitation. The Young's modulus was resolved using the DMT's contact model.

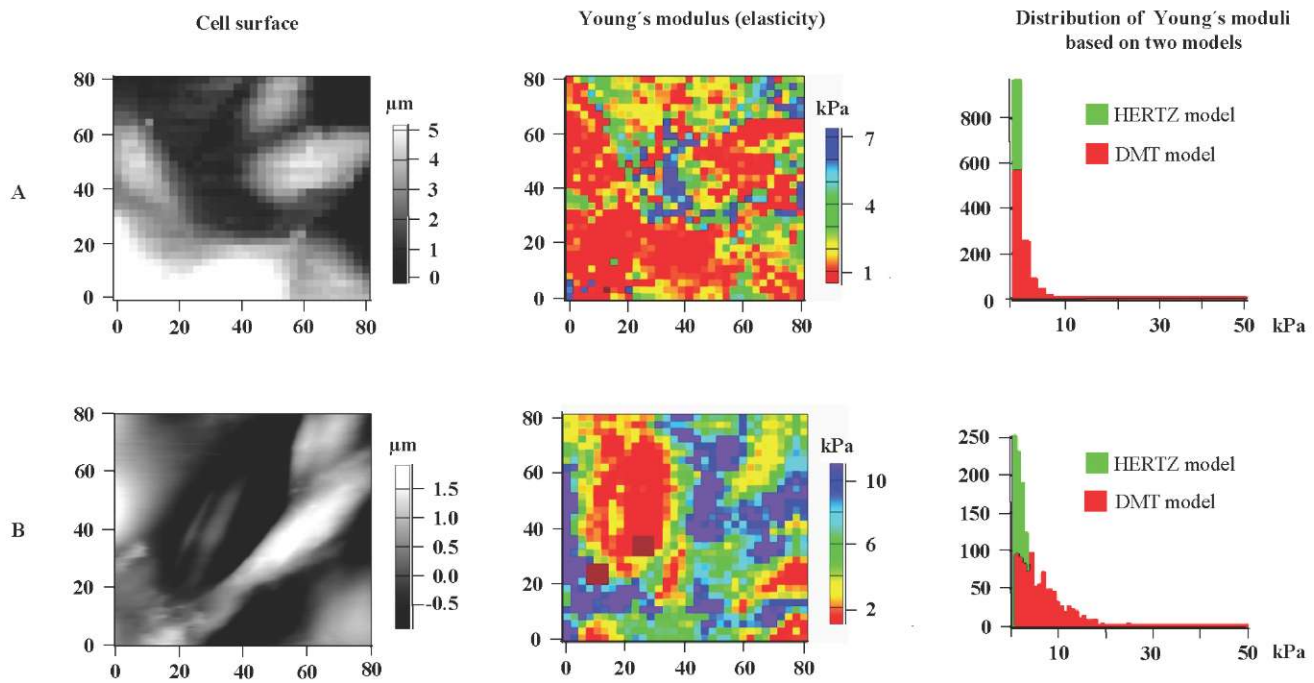

Figure 1. Effect of 6EOPA on the nanomechanical parameters of the heart cells surfaces measured with atomic force microscope technique. Young's modulus characterizes the elasticity of the cells, which is moderately changed in the presence of low ophiobolin concentration. 6EOPA:6-epi-ophiobolin A; A: Control cells, B: 6EOPA treated cells..

Results

The membrane surface tension was investigated by AFM method and the distribution of kv4.2 and kv4.3 ion channels in the membrane of H9c2 heart cell line was investigated using immunofluorescence labeling.

Effect of 6-epi-ophiobolin A on mechanical parameters of cardiomyocytes

The effect of 6EOPA treatment on the nanomechanical properties of membrane surface of rat cardiomyocytes was measured with AFM method (Fig. 1). The heights of cells, the stiffness of the membrane and the elasticity were investigated. Elasticity was characterized with Young's modulus of the cell surface, which was changed moderately after adding low concentration 6EOPA.

Heights of cardiomyocytes were calculated by two models, both were demonstrated the average heights of cells in microscope. The height of cardiomyocytes was altered mildly and the values of Hertz and DMT model for plasma membrane (PM) are shown in Figure 1. Both micrographs and the calculated Young's modulus figures indicated the different topology of surface for healthy and treated myocytes. This was confirmed by kPa values in Hertz and DMT model, where these values were increased by about 50% for each treated cell compared to healthy controls presenting with representative figure.

PM of cardiomyocytes was characterized by mechanical parameters in the presence of 6EOPA. Young's modulus characterizes the elasticity of the cell surface, which is moderately changed in the presence of low ophiobolin concentration (Fig. 1).

Environmental effects on kv4.2 and kv4.3 ion channels

Specifically labeled kv4.2 and kv4.3 channels were studied on adult rat cardiomyocytes in healthy cell line and 6EOPA treated cells. These experiments were performed parallel with AFM measuring with low concentration of 6EOPA.

Kv4.2 and Sap97 channel proteins were expressed abundantly in the PM of healthy cells and they colocalized at the lateral side of PM and around nuclear membrane (Fig. 2). Kv4.2 channels were labeled fainter at the lateral side. Decreased number of kv4.2 channels was detected in the PM, also the cytoplasmic area in treated cells with HMGB1

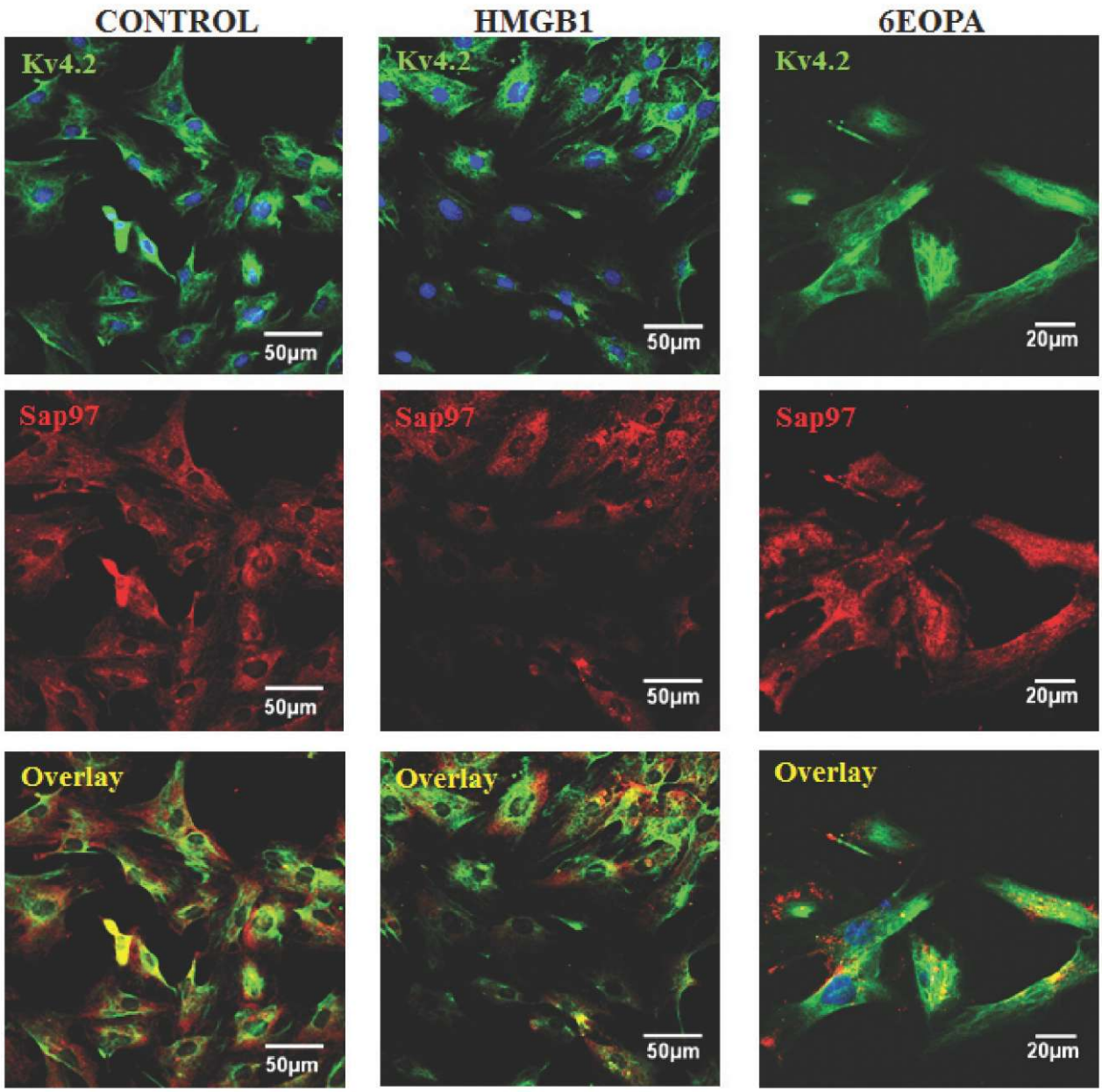

Figure 2. Immunofluorescent staining of kv4.2 ion channel after 6EOPA or HMGB1 treatments in rat heart cells. Kv4.2 ion channel colocalizes with Sap97 anchoring protein in the plasma membrane and in cytoplasm of cardiomyocytes. This association and distribution were altered after 6 EOPA or HMGB1 treatment in rat heart cells. 6 EOPA: 6-epi-ophiobolin A; HMGB1: high mobility binding protein 1; Sap97: Synaptic associated protein 97.

or 6EOPA. The amount of Sap97 protein was decreased and colocalization could not be detected with kv4.2 protein in the presence of HMGB1, while some colocalization was visible after 6EOPA treatment in few cells (Fig. 2).

To examine the 6EOPA or HMGB1 effect on kv4.3 channels, the same expanded cell cultures were used. Kv4.3 ion channels have strong labeling on the PM and on the membrane of the nucleus colocalized with Sap97 proteins (Fig. 3). Following treatment with 6EOPA, less amount of kv4.2 and kv4.3 channels were detected around the nucleus (Figs. 2, 3). The labeling was shown little faint sign in the PM, too.

The same effects were detected on kv4.2 and kv4.3 chan-

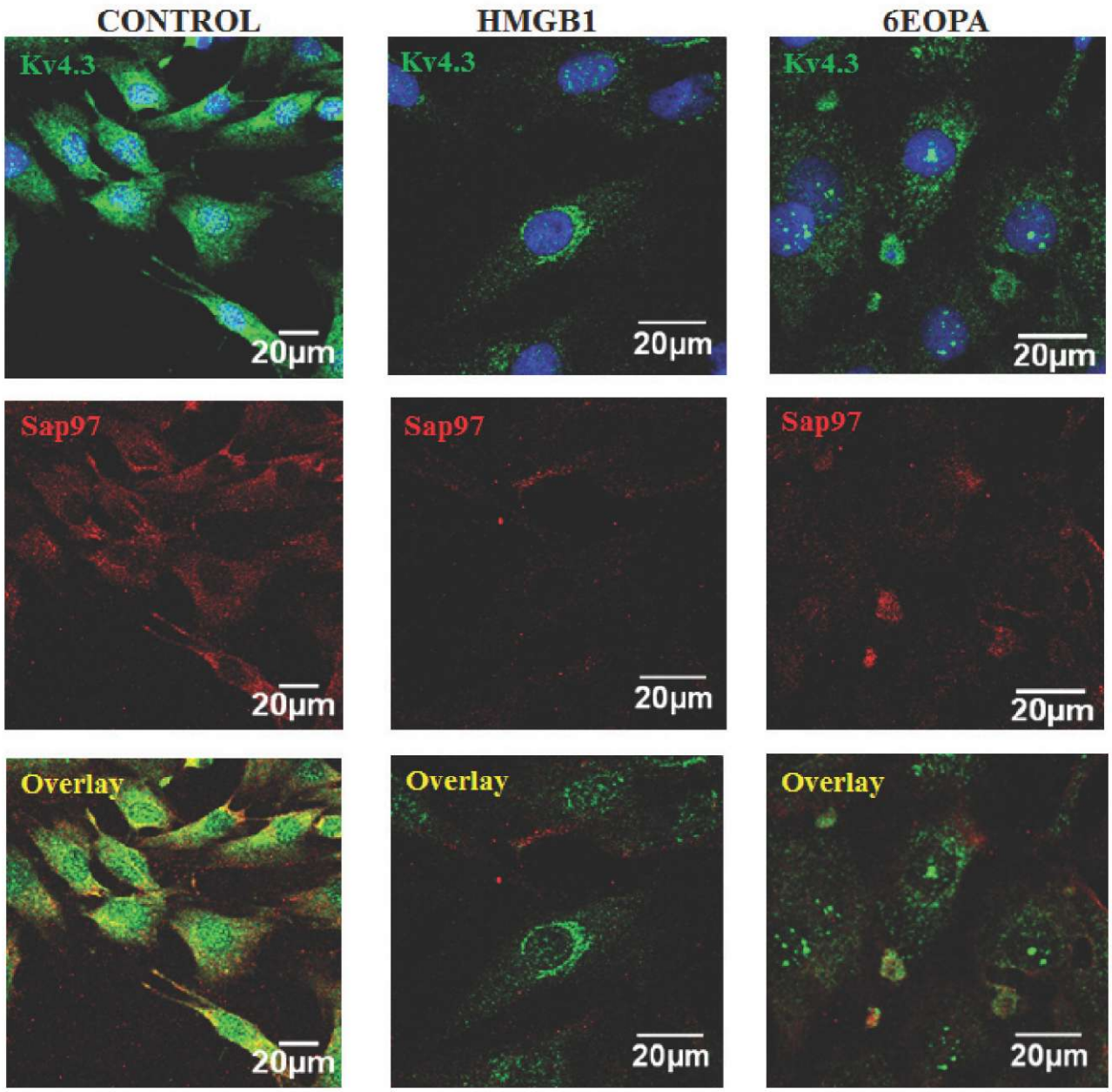

Figure 3. Immunofluorescent staining of kv4.3 ion channel after 6EOPA or HMGB1 treatments in rat heart cells. Kv4.3 ion channel and Sap97 protein were colocalized partially in the presence of 6EOPA or HMGB1 in cardiomyocytes. 6EOPA, 6-epi-ophiobolin A; HMGB1, high mobility binding protein 1; Sap97: Synaptic associated protein 97.

nels in the treatment of HMGB1 or 6EOPA. Effect of low concentration of 6EOPA was exerted on kv4.2 and kv4.3 channels.

Colocalization of Sap97 with kv4.2 after HMGB1 or 6EOPA treatment decreased or could not be detected in rat heart cells.

We were interested in the alteration of expressed kv4.x channels and distribution with Sap97 in cardiomyocytes after external stress and wether 6EOPA influences this colocalization. The high intensity shows the abundant kv4.3 in the control rat cardiomyocytes overlapping with Sap97. It changed upon adding stress factors HMGB1 as a cytokine and 6EOPA, respectively. The presence of HMGB1 or 6EOPA exerted the same down-regulation on the kv4.3 channel complexes.

Discussion

To address the importance of potassium channels, we investigated the effects of the 6EOPA as external stress factor and HMGB1 as a cytokine on kv4.x channels in heart cells. Novel results are: (1) The kv4.3 ion channels localized mainly around the PM and on the membrane surface as well as on the nuclear membrane of rat cardiomyocytes. (2) Distribution and amounts of kv4.2 and kv4.3 ion channel complexes changed in the presence of 6EOPA and HMGB1 in cardiomyocytes. (3) Immunostaining revealed that Sap97 co-localization with kv4.3 ion channel α-subunits altered in the presence of 6EOPA at the sarcolemma and may also change in other subcellular organs compared to normal healthy cardiomyocytes. AFM measurements were used to obtain new information from cell surface alteration caused by OPA derivatives on the cardiomyocytes. AFM makes possible to investigate membrane rigidity caused by severe heart or muscle diseases, leading to reversible and irreversible changes on the cardiomyocytes. The results of AFM had an opportunity adapting methods, directly on living cells, which is the most human-like cardiomyocyte model. The examination of this cell model may help to find an appropriate drug to develop new methods for testing effective treatments in diseases.

AFM study showed that the Young's modulus and the mechanical parameters are altered on the cell surface after one day in the presence of 6EOPA. In this investigation, the kPa values in Hertz and DMT model indicate the altered nanomechanical parameters for mild effects on or in the PM. In adult cardiomyocytes, increased heights were measured suggesting the different structure of PM from healthy cells, which could be even reversible due to the used low concentration of 6EOPA tolerating the applied circumstances. These data may not be applicable for altered parameters of PM and the other biophysical and biochemical background in acute treatment, only when the latest method, the combination of AFM with patch-clamp study could be adapted (Ossola et al. 2015; Chen et al. 2016).

Remodeling of ion channel expression and function may be the result of modulation of gene transcription, mRNA processing, mRNA translation, protein processing, subunit assembly, membrane transport, assembly into macromolecular complexes and posttranslational regulation. The understanding of this remodeling and the difference between physiological and pathological adaptation will play an important role in the future therapeutic measures against cardiac disease (Rosati and McKinnon 2004). Kv4.x channel protein expression was demonstrated in the intercalated discs in heart tissues (Chen et al. 2016) and supported by earlier studies showing the decreased or increased amount of kv4.x ion channels with western blotting methods in diseased heart cells, which the effects of different drugs (Bányász et al. 2011; Jost et al. 2013; Gómez-Hurtado et al. 2014). In our experiments, down-regulation and redistribution of kv4.2 and kv4.3 ion channels were revealed in the PM after the 6EOPA treatments.

Recently, it was shown that HMGB1 can serve as a proinflammatory marker in tissues, where inflammation caused changes after treatment (Yang et al. 2005, Liu et al. 2010). This highly conserved protein is present in the nuclei and cytoplasm almost in all cell types, but the administration of high amount of HMGB1 to healthy animals causes inflammatory effects including fever, weight loss, anorexia, lung injury, epithelial barrier dysfunction, arthritis and death. The opposite effect, down-regulation of HMGB1 has been shown to inhibit autophagy using lycorine administration in multiple myeloma (Roy et al. 2016).

Our investigations demonstrated the exogenous toxic effects of administrated 6EOPA or high concentration of HMGB1 on kv4.x ion channel complexes anchoring with protein Sap97 in cardiomyocytes. Sap97 protein did not co-localize with kv4.x ion channel α-subunits at the sarcolemma in the presence of 6EOPA or HMGB1 compared to the controls indicating that the anchoring function was disturbed. Sap97 and kv4.x channel subunits co-localize at the sarcolemma of healthy cardiomyocytes, but the distribution of these complexes has changed by treatment of a derivative of 6EOPA. Furthermore, our experiments suggested that HMGB1 treatment may damage also the colocalization of Sap97 with kv4.x channels in all membranes of cell organs as well as in the PM. The results of the present study indicated that the cardiotoxic effects disrupted the ion harmonization in the cells and perhaps the membrane structure was involved in this process.

Earlier the kv4.2 and kv4.3 channels have been detected in the intercalated discs using immunofluorescence methods of healthy cardiomyocytes (Roepke et al. 2008; Foeger et al. 2013; Liu et al. 2015). In our experiments the kv4.2 ion channel expression in healthy rat cardiomyocytes was abundant in the cytoplasm area. The Sap97 anchoring protein, in our experiments, showed the same distribution and co-localization with kv4.2 channels in the cytoplasm of myocytes as well as kv4.3 in healthy myocytes. These results are the first evidence for redistributed kv4.2 as well as the kv4.3 ion channels in the presence of OPA derivative or HMGB1 as exogenous cytokine.

Liu et al. have shown (2010) that the effect of HMGB1 treatment for 24 h: significantly inhibited both the current densities of heterologously expressed kv4.3 and v4.2 in COS-7 cells and the native I_{to} in neonatal rat ventricular myocytes (NRVMs) in a dose-dependent manner. The increased level of HMGB1 in the cytoplasm decreased both the mRNA and protein levels of the α-subunits kv4.2 and kv4.3 channels. We suppose that the 6EOPA treatment changed the heterologous expression of kv4.x channels in PM. Altered kv4.x pattern may be related to the biological activity and

cytotoxicity of some exogenous compound, leads to NF-mediated response under stress effects (Chan et al. 2012; Oeckinghaus et al. 2011).

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ARTICLE

Nutrient content in leaves of hydroponic lettuce (*Lactuca sativa* convar. *capitata* L.) on higher magnesium and nitrogen nutrient treatment

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ABSTRACT The aim of this study was to assess how the steadily increasing concentration of a physiologically important element, magnesium, can affect the content of certain nutritional elements in the leaves of the hydroponic cultivated lettuce (*Lactuca sativa* convar. *capitata* L.). The nutrient elements nitrogen, magnesium and calcium were determined in the leaves of lettuce after supplementation with magnesium. Increasing magnesium concentration in the nutrient solution caused decrease of nitrogen content of dry matter in the leaves from 4.94 m/m% to 4.39 m/m%. The concentration of magnesium increased from 0.303 m/m% to 0.571 m/m%, and that of calcium decreased from 0.723 m/m% to 0.358 m/m%, in the average of 4 repetitions. Increasing concentration of magnesium can be the consequence of increased amounts of magnesium supplemented while decrease of nitrogen and calcium can be explained by the phenomenon of attenuation. **Acta Biol Szeged 60(2):167-169 (2016)**

KEY WORDS

hydroponic cultivation
Lactuca sativa
lettuce
nutrients

Introduction

Lettuces are grown in extraordinary wide range of varieties, and by now turned from a seasonal vegetable into an all-year-round grown food. Modern technologies, such as hydroponic growing, permit continuous cultivation of lettuce for 12 months of the year. Average consumption of this plant increased during the previous decade because it fits into modern healthy nutrition. Lettuce (*Lactuca sativa* convar. *capitata* L.) is a significant, vitamin-rich vegetable. In growing without soil, lettuce shows several advantages such as faster development, higher average yield, balanced and scheduled development; furthermore, growing can be automated which is environment friendly and does not require significant manual labour (Morgan 1999).

Magnesium is a central component of chlorophyll, which has an essential role in photosynthesis; magnesium is also an essential metal in the plant metabolism, protein biosynthesis, and collaborates as a metallic catalyst in take and release of energy (Somos et al. 1980; Somos 1983; Terbe and Fehér 2000; Terbe et al. 2001; Terbe 2007; Terbe et al. 2011).

The temperature and humidity of the greenhouse can be easily controlled with the automated system. The nutrient solution can also be circulated multiple times a day. The advantage of the technology is that the plants grow at the same rate and the heads are harvested at the same time. Harvesting is a short and smooth process, so it makes an efficient use of human resources. This method can be used repeatedly, allowing continuous production throughout the year. Lettuce can be grown as much as six times on rock wool in a year.

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Materials and Methods

Hydroponic experiment was conducted in the greenhouse of the Faculty of Horticulture of Kecskemét College (Hungary) at the end of September 2014. The lettuce seedlings were placed into rock wool cubes and put into hydroponic growing channels. In the experiment, the standard nutriment solution was supplemented with magnesium, in form of $Mg(NO_3)_2$ solution, in the doses of 50, 100, 150, 200 and 250 mg/l. In the control, plants were grown with standard nutriment solution.

The standard nutriment solution was made from the following water-soluble fertilizers (only highly soluble fertilizers were used): 666.7 g Ferticare komplex (N 14%, P_2O_5 11%, K_2O 25%); 733.3 g $Ca(NO_3)_2$ (N 15%, CaO 26%); 66.7 g KH_2PO_4 (P_2O_5 54%, K_2O 32%); 100 ml 60 m/v% H_3PO_4 added to 1000 l of water. The standard solution contained both magnesium and microelements: 14.7 g MgO; 0.67 g Zn, Cu, Fe, Mn; 0.13 g B; 0.013 g Mo per 1000 l of solution.

Table 1. Lettuce head weight measured after Mg supplementation. Results are means ± SD of five measurements. For statistical analysis Tukey HSD test was used.

Treatments	Mean (g)	Standard deviation	Significance level
Control	285.93	21.106	-
50 mg/l	231.79	31.123	0.000
100 mg/l	257.61	20.058	0.001
150 mg/l	205.18	30.804	0.000
200 mg/l	217.57	24.309	0.000
250 mg/l	203.86	23.418	0.000

The hydroponic tanks of 28 l volume were filled with fresh nutrient solution every week. In the hydroponic system watering with the nutrient solution was done using an automated pump. Circulation of the nutrient solution was started for 15 min at 10 and 14 o'clock.

Experimental plants were propagated by seeding. Seedlings were transplanted to multi-cell transplant raising trays on 3rd September, and the lettuce seedlings were placed into rock wool cubes, and put into hydroponic growing channels on 17th September. Each channel of the closed nutrient system had a separate container with a separate submersible pump to ensure adequate circulation of the nutrient solution for plants. The number of plants per plot was 28. The experimental design was a randomized blocks with 4 repetitions. Temperature in the greenhouse was between 15 and 20 °C. No chemicals or herbicides were applied. The first harvest took place on 14th November, and the biomass was recorded by measuring 7 plants from each treatment group.

Analytical tests were performed in the Soil and Plant Testing Laboratory of Faculty of Horticulture and Rural Development (Pallasz Athéné University, Kecskemét, Hungary). Standard analytical methods were used. Lettuce leaves were dried in LTE-OP-250 drying oven at 70 °C for 48 h and then were homogenized and digested by wet destruction. Total amount of nitrogen was tested by Kjeldahl method. Magnesium and calcium content of the samples were analyzed by optical emission spectrometer (ICP-OES method) (Mindak et al. 2014).

Electrical conductivity of the nutrient solutions was measured by laboratory EC-meter (type ORION 3Star) on 13th October in two repetitions, in two growing channels, respectively. Statistical analysis was done with SPSS v19 software (Huzsvai 2004). The mean difference was regarded significant at the 0.05 level ($P \leq 0.05$).

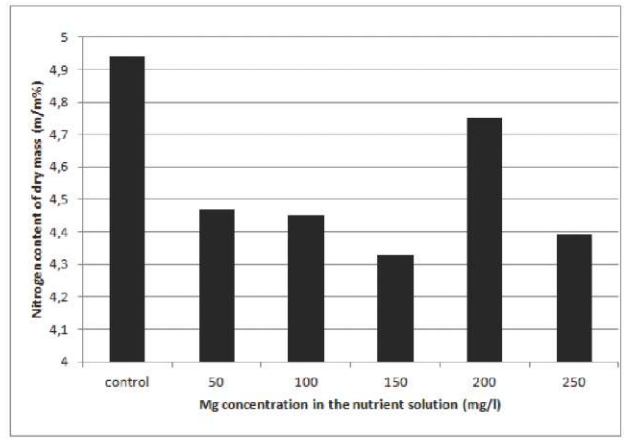

Figure 1. Nitrogen content of dry matter in the lettuce leaves (m/m%) after supplementation with Mg.

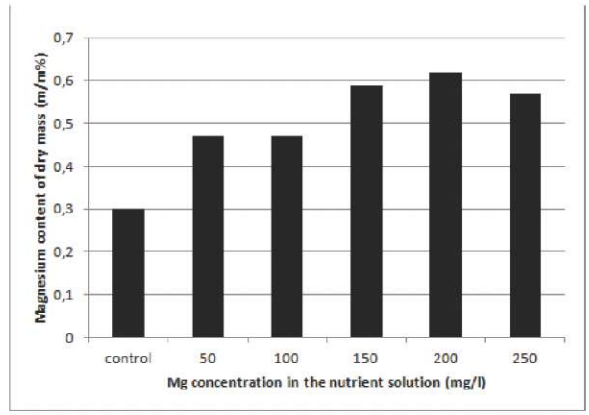

Figure 2. Magnesium content of dry matter in the lettuce leaves (m/m%) after supplementation with Mg.

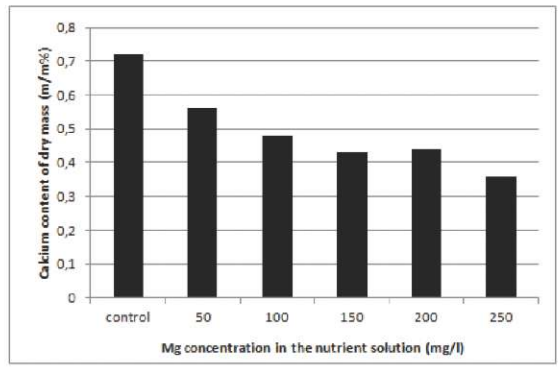

Figure 3. Calcium content of dry matter in the lettuce leaves (m/m%) after supplementation with Mg.

Results

For our statistics calculations we compared the growth of the Mg-treated lettuce to that of the control plants. Data about lettuce head mean weight are shown in Table 1.

We measured 28 plants from each treatment area. Minimum and maximum mass can be found in Table 1. The maxi-

mum weight was found in the control group, while the minimum was among the 150 mg/l and 250 mg/l treatments.

Nutrient element concentrations of nitrogen, magnesium and calcium were determined in the leaves of lettuce. Increasing magnesium concentration in the nutriment solution caused decrease of nitrogen content of dry matter in the leaves from 4.94 m/m% to 4.39 m/m% (Fig. 1). Magnesium concentration increased from 0.303 m/m% to 0.571 m/m% (Fig. 2), and calcium decreased from 0.723 m/m% to 0.358 m/m% (Fig. 3), in the average of 4 repetitions. Increasing concentration of magnesium can be the consequence of magnesium supplementation, while decrease of nitrogen and calcium concentrations can be explained by the phenomenon of dilution effect.

Discussion

Lettuce was grown using hydroponic cultivation in our study. Their growth was steady, but there was a great deviation in head weight, that is, magnesium treatment led to a significant decrease in head weight compared to control. The heaviest heads were found among the control plants (285.93 g), and the second heaviest, in the 100 mg/l treatment (257.64 g). Applying 200 mg/l magnesium led to 24% decrease, and 50 mg/l, to 19% decrease of lettuce head weight compared to the control. The effect of 250 mg/l and 150 mg/l was similar to each other.

Possibly due to a kind of antagonism, magnesium concentration blocked potassium uptake and thus led to the large differences in mass. Potassium is a mobile, translocating element and so the deficiency symptoms were first observed on the older leaves. Potassium deficient plants have reduced disease resistance, chlorosis and necrosis often occurs later in lettuce. Calcium deficiency symptoms were also noticeable. The roots had insufficient growth, the apices were mucous, turned brown, and subsequent necrosis was observed. In some cases the growth of the plants failed to begin as the lettuce did not take up the nutrients at a proper rate.

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ARTICLE

Antioxidant, physicochemical and mineral evaluations of *Spondias mombin* crude fruit juice

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ABSTRACT Edible fruits have potential health benefits regarding their richness in minerals, nutrients, vitamins and antioxidant. In view of the medicinal potential of the leaves and stem bark extracts of *Spondias mombin*, this study aimed to determine the antioxidant, physicochemical and mineral contents of the crude fruit juice of *S. mombin* for its employment in health promotion and traditional use for medicine. Free radical scavenging power of 93.97 ± 64.8 µmol (TE) and Ferric reducing power (FRAP) of 11.8 ± 0.2 µmol (AAE) were observed *in vitro* from the crude fruit juice. The physicochemical property of the crude fruit juice yielded proximate compositions of protein (6.03 ± 0.44), fat (1.85 ± 0.03), pH (3.63 ± 0.11), fibre (0.64 ± 0.02), moisture (80.60 ± 0.22%), ash (0.54 ± 0.03), carbohydrate (11.61 ± 0.34) and titratable acidity (28.52 ± 0.14). Trace elements from the crude fruit juice are in the amounts of 276.27 ± 2.65 for potassium, 1.15±0.01 (manganese), 0.121 ± 0.32 (copper), 4.45 ± 1.42 (sodium), 136.42 ± 1.35 (zinc), 12.36 ± 0.03 (magnesium), 0.01 ± 0.00 (lead), 28.22 ± 1.02 (phosphorus), 13.04 ± 0.53 (calcium) and 0.48 ± 0.01 (iron).

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KEY WORDS

antioxidant
fruit juice
physicochemical
Spondias mombin

Introduction

In Nigeria, large numbers of underutilized wild fruits are widely distributed throughout the country. Most of these wild fruits are consumed mainly by rural duelers, who appreciate them in quenching of thirst and supplement for food without knowledge about their health importance and medicinal values. The Nigeria climate has favorable potency for the production of tropical fruits. These tropical fruits are seasonal and some are cultivated for their health benefits, while others are readily available as wild in the forests uncultivated by man. Fruits are important sources of many nutrients and have served as integral part of the human diet as they supply vitamins, minerals and other vital constituents essential for human health (Akhtar et al. 2010). Fruits are believed to occupy a modest place as a source of trace elements due to their high water content. However, consumers look for variety in their diets and therefore opt for fresh fruits because of the health benefits. Fruits are special food sources that are rich in potassium (K), calcium (Ca) and magnesium (Mg). These minerals among others are nutrient requirements of which their intake is associated with reduced risk of, *e.g.*, cardiovascular diseases, cataracts, and age-related functional decline (Willett 1994, 1995; Temple 2000; Aberoumand and Deokule 2010). Higher amounts of antioxidants such as vitamin A, C, E, lycopene, polyphenols and carotene are present in fresh fruits, than when prepared into juice and beverages these components are often reduced.

Numerous studies have shown that fruits are rich sources of nutrients, as well as non-nutrient molecules with antioxidants or other physiological effects that are important constituents of a healthy diet. The health promoting properties of plant-based foods have largely been attributed to their wide range of phytochemicals, of which many are present at relatively high levels (Ali et al. 2011).

Normal physiological processes *in vivo* result in the production of free radicals. Oxidative stress created when there is an insufficient capacity of the biological system to neutralize excess free radicals. This may result in aging and disease conditions as reported by Sahreen et al. (2014). Fruits and other foods are known to contain antioxidants that are linked to *in vivo* protection from oxidative stress (Jensen et al. 2008). Different studies have shown that free radicals present in the human organs, cause oxidative damage to various bio-molecules, such as lipids, proteins and nucleic acids, and

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thus are involved in the initiation phase of certain degenerative diseases. Phenolic and other phytochemical antioxidants found in fruits and vegetables capable of neutralizing free radicals and may play a major role in the prevention of certain diseases (Kaur and Kapoor 2001).

Numerous epidemiological investigations have pointed out that a lack of essential mineral elements in food can result an increase in sensitivity to illness, leading to suboptimal health or an enhancement of disease occurrence. Among others iodine, selenium, zinc, iron, copper, manganese and chromium were recognized as essential mineral elements indispensable for maintaining normal life activities. All these mineral elements can be obtained from the consumption of certain foods. It was also recognized decades ago that green and natural foods of plant origin are of important sources of these minerals. These metals are commonly found in trace amounts in various plants as they easily take up them from the environment. At the same time, different parts of a plant could contain different concentration of these elements. In spinach, *e.g.*, the amount of Mn in the tip of the leaf was higher than in the other parts, or Co and Zn were mainly accumulated around the main veins (Xin et al. 2009).

Spondias mombin is a tree having habitat in several tropical forests in the world. In Nigeria, it is widely distributed in all geographical regions as wild plant. It is called *Iyeye* by the Yorubas, Ichikara, or *Uvuru* by the Ibos, and *Tsaadar lamarudu* by the Hausas. Names of this plant in some other parts of the world are *Bala* (Costa Rica), *Jobito* (Panama), *Jobo blanco* (Colombia), *Jobo corronchoso* (Venezuala). *Hoeboe* (Surinam), *Acaiba*, *Caja*, *Pau da taperra* (Brazil, Ubo (Peru), and *Hobo* (Mexico). For its ethno-medicinal uses, the fruit juice is consumed as a diuretic, febrifuge and laxative. The aims of this study were to analyze the antioxidant activity, physicochemical properties and mineral composition of *S. mombin*. The long-term goal is to promote and increase the utilization and consumption of edible wild fruits.

Materials and Methods

Collection and preparation of fruit juice

Ripe fruits of *S. mombin* were harvested from its tree at the town of Ado Ekiti (Nigeria). The fruit was identified by a botanist in the Department of Biological Sciences, Afe Babalola University (Ado Ekiti, Nigeria). The fruits were washed with 3% sodium hypochlorite and rinsed severally in distilled water. The fruits were pressed for juice to be extracted. The extracted crude juice was filtered through No 1 Whatman filter paper and the filtered juice was kept in a sterile bottle at 4 °C before use.

Determination of radical scavenging capacity with 1,1-diphenyl-2-picrylhydrazyl (DPPH)

DPPH radical scavenging activity was determined according to Cavin et al. (1998). Two milliliters of the crude fruit juice was added to 1 ml of DPPH solution (0.04 mg/ml in methanol). The mixture was vigorously shaken and incubated in the dark for 20 min. Thereafter, the reduction of DPPH absorption was measured at 517 nm. A calibration curve was prepared by measuring the reduction in absorbance of the DPPH solution in the presence of different concentrations of Trolox (0-400 µM). Results were expressed as µmol of Trolox equivalents (TE)/mg wet extract. All determinations were performed in triplicate.

Determination of ferric reducing activity (FRAP)

The ferric reducing activity of the fruit juice was estimated based on the FRAP assay (Benzie and Strain 1996). The solutions for this assay are 300 mmol/L acetate buffer, 10 mmol/L TPTZ (2,4,6- tris(2-pyridyl)-s-triazine) in 40 mmol/L of HCl and 20 mmol/L $FeCl_3$ (6 H_2O). From this 25 ml acetate buffer was mixed with 2.5 ml of TPTZ solution and 2.5 ml $FeCl_3$ (6 H_2O). The assay was performed by adding 1 ml of the FRAP reagent to 2 ml of diluted fruit juice. Absorbance reading at 593 nm was recorded after 20 min of the reaction. The change in absorbance was related to the absorbance change in ascorbic acid as the standard solution tested in parallel. Results were expressed as micromole of ascorbic acid equivalents (AAE)/mg of wet weight of the fruit juice. All determinations were performed in triplicate.

Determination of total phenol

Total phenol concentration of the crude fruit juice was determined by spectrophotometric method, according to the Folin-Ciocalteu colorimetric method of Singleton and Rossi (1965), using caffeic acid as the standard and expressing the results as µmol equivalents of caffeic acid (CAE)/mg of wet weight of fruit juice.

Determination of total flavonoids content

Total flavonoids from the crude fruit juice were estimated by colorimetric method using the criteria of Sakanaka et al. (2005). Aliquot of fruit juice was mixed with 400 µl of distilled water and 30 µl of a 5% sodium nitrite solution. After 6 min, 10% aluminium chloride solution (30 µl) was added and the mixture was allowed to stand for 5 min. Then 200 µl of 1M NaOH solution and 240 µl of distilled water were added and the absorbance was measured at 510 nm. Rutin was used as standard solutions. All determinations were done in

Table 1. Antioxidant profile of the crude fruit juice of *S. mombin*.

Antioxidant	Mean ± SD
Free radical scavenging activity (DPPH) (µmol TE)	93.97 ± 64.8
Ferric reducing activity (FRAP) (µmol AAE)	11.8 ± 0.24
Total flavonoids (RE/mg wet fruit juice)	14.5 ± 0.04
Total phenol (CAE/mg wet weight fruit juice)	155.126 ± 12.546

triplicate and values were calculated from a calibration curve obtained with rutin. Final results were expressed as µmol of rutin equivalents (RE)/mg wet fruit juice.

Determination of physicochemical properties

The standard methods of Association of Official Analytical Chemists, (AOAC 2005) was used to analyze the physicochemical properties of the *S. mombin* crude fruit juice. All analysis were made in triplicate.

Determination of mineral contents

Wet ashing method was used to digest the crude fruit juice and the quantification was by atomic spectroscopy and mass spectrometry (ICP-MS, Spectro Analytical Instruments GmbH, Germany) according to the AOAC methods 997.15 and 990.08 (AOAC 2005). The results were expressed in mg/100 g. All analysis were performed in triplicate.

Statistical analysis

The results were expressed as mean ± standard deviation (SD) and subjected to one way analysis of variance (ANOVA). The least significant difference (LSD) was performed for the pairwise mean comparisons to determine the significant treatment dose at 95% level of confidence. Values were considered statistically significant at (P<0.05).

Results and Discussion

Antioxidants capacity of the crude fruit juice of S. mombin

Free radical scavenging power of 93.97 ± 64.8 µmol (TE) and ferric reducing power (FRAP) of 11.8 ± 0.2 µmol (AAE) were measured as reasonable amounts of the juice ability in *in vitro* antioxidant capacity (Table 1). The total antioxidant activity shown by the crude fruit juice of *S. mombin* reflects substantial free radical scavenging ability and reductive potential.

Free radicals are frequently generated in the living system and could be responsible for cell and tissues damage. As a consequence, research is focused on exploring for safe and effective antioxidants and also to encourage the consumption of natural antioxidants from food supplements and traditional medicines (Yazdanparast and Ardestani 2007). Antioxidant compounds, such as phenolic acids, carotenes and vitamins have been reported to be naturally present in fruits, vegetables, nuts, herbs and spices (Ali et al. 2008; Vasco et al. 2008; Schinella et al. 2009; Sreeramulu and Raghunata 2010; Lu et al. 2011). The results obtained in this study, indicates that the crude fruit juice of *S. mombin* is a good source of antioxidants, that could be of help to alleviate oxidative stress. Based on the evaluated antioxidant properties by Adewale et al. (2015), Famobuwa et al. (2016a; 2016b) on some Nigerian fruits, the result obtained allows us the insight that the crude fruit juice of *S. mombin* possessed antioxidant activity above average. Along with some fruits, such as pineapple, banana, watermelon, and orange and vegetables such as tomato and carrot, that are commonly consumed on daily bases by the populace. Also, the report of free radical scavenging activity (71.5%) and ferric reducing potential (1.94%) from the leaves of *S. mombin* as reported by Awogbindin et al. (2014) were less value to what was obtained from the crude fruit juice of *S. mombin* in this study.

The amounts of phenolic compounds and flavonoids contents of *S. mombin* in this study suggest its high antioxidant capacity. Phenolic compounds of plants fall into several categories; chief among these are the flavonoids, which have potent antioxidant activities (Tadhani et al. 2007). Flavonoids derivatives have shown a wide range of antibacterial, antiviral, anti inflammatory, anticancer, and anti-allergic activities (Di Carlo et al. 1999; Montoro et al. 2005). Flavonoids, have been shown to be highly effective scavengers of most oxidizing molecules, including singlet oxygen and various free radicals implicated in several diseases (Nabayi et al. 2008).

As *S.mombin* fruit juice exhibited significant levels of phytochemicals, which make it a potent source of antioxidant agents. This fruits can be vital help especially in such rural and urban areas where modern health facilities are not accessible due to poor state of living. Recent studies have also shown the advantages of natural antioxidants in foods, as opposed to synthetic additives (Shalini 2012).

Physicochemical properties of the fruit juice of S. mombin

The most important physicochemical properties of the crude *S. mombin* fruit juice were also determined. Thes values are displayed in Table 2.

The nutritional and energy values of foods are known through proximate analysis, but this gives a certain insight to the food quality. Earlier, Adepuju (2009) reported higher values for Nigerian *S. mombin* fruit juice (g/100 g) in moisture (82.3), lipid (2.0), crude fibre (4.2) and ash (1.0) then our values. From Brazil, Tiburski and co-workers (2011) reported moisture (83.66), fat (0.62), fiber (1.87), ash (0.76), total acidity (20.85), protein (1.06), carbohydrate (13.90) and pH (2.83) values, which are not significantly different from those we measured in this study.

Mineral composition of the crude fruit juice of S. mombin

Ten trace elements (potassium, manganese, cupper, sodium, zinc, magnesium, lead, phosphorus, calcium, and iron) were determined from the crude fruit juice of *S. mombin*. The measured concentrations are displayed in Table 3. Some of these values are higher than what are reported for apricots, apple, banana, blackberry, grape fruit, cherries, lemon, lime, pear, pineapple, watermelon and other common and frequently consumed fruits (Cunningham et al. 2001; Emsley 2001; Decuypere 2005).

Minerals or trace elements are either essential or non-essential, depending on whether or not they are required for human nutrition and have metabolic roles in the body (Reilly 2002). Fruits generally have in their composition a great variety of vitamins and essential minerals, which makes them beneficial to our diet. In this study, low levels of manganese, iron, sodium, copper and lead were observed in the crude *S. mombin* fruit juice though higher content of magnesium, phosphorus, potassium and zinc have been reported present in some other edible fruits. Albino et al. (1999), described *S. mombin* as a fruit with high content of potassium, along with jackfruit, soursop, jenipapo (*Genipa americana*) and mangaba (*Hancornia speciosa*). The phosphorus content of *S. mombin* as found in this study is one of the highest among the commonly consume fruits with levels close to those of Ceriguela (*Spondias purpurea*), Pequi (*Caryocar brasiliense*) and Passion fruit (NEPA-UNICAMP 2006).

Same mineral contents evaluated from *S. mombin* fruit juice by Adepoju (2009), cannot be compared because of the different methods used in analysis. Though several researchers have evaluated and reported different mineral contents of *S. mombin*, the differences can be attributed to the origin of the fruit. It may be dependent on the environmental conditions of each region (Leterme et al. 2006), since the minerals are absorbed from the soil. Furthermore, genetic factors, the use of fertilizers (Sanchez-Castillo et al. 1998) and the maturity stage of the tree that produces the fruits could also affect these results. The mineral contents of sodium, magnesium, potassium and phosphorus reported by Tiburski et al. (2011) from Brazil, were a bit higher than what was obtained in our study, while we measured higher calcium, manganese, iron, copper, lead, and zinc levels.

Table 2. Physicochemical properties of the fruit juice of *S. mombin*.

Physicochemical properties	Mean ± SD
pH	3.63 ± 0.01
Moisture (g/100 g)	80.66 ± 0.02
Protein (g/100 g)	6.03 ± 0.04
Fiber (g/100 g)	0.64 ± 0.02
Ash (g/100 g)	0.54 ± 0.03
Fat (g/100 g)	1.85 ± 0.02
Total titratable acidity	28.52 ± 0.04
Carbohydrate (g/100 g)	11.61 ± 0.04

Table 3. Mineral content of the crude fruit juice of *S. mombin*.

Minerals	Mean ± SD (mg/100 g)
Sodium (Na)	4.45 ± 1.42
Magnesium (Mg)	12.36 ± 0.03
Phosphorous (P)	28.22 ± 1.02
Potassium (K)	276.27 ± 2.65
Calcium (Ca)	13.04 ± 0.53
Manganese (Mn)	0.15 ± 0.01
Iron (Fe)	0.48 ± 0.01
Copper (Cu)	0.121 ± 0.32
Lead (Pb)	0.01 ± 0.00
Zinc (Zn)	136.42 ± 1.35

Conclusions

The level of antioxidant in fresh fruits are in wholesome than the preserved in the commercial fruit juices. Many of these compounds are heat sensitive, which are destroyed during the industrial processing of juices. Additionally, these antioxidants frequently used for preservative purposes and are synthetic additives in the product. We are of the opinion that consumption of fresh *S. mombin* fruit juice will promote a better health status as the antioxidants, physicochemicals and minerals analyzed are vital and valuable than some of the many recognized fruits. The analysis of the crude fruit juice of *S. mombin* in this study is in support of the several reports of health benefits and medicinal value of this edible fruit.

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ARTICLE

Antioxidant profile of tomato landraces for fresh consumption

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ABSTRACT Tomato is one of the most important and frequently consumed vegetable species in Hungary, as well as a significant vitamin source throughout the year. Due to its antioxidant content, tomato consumption is related to the reduced occurrence of cardiovascular diseases and certain cancer types. In our study, four Hungarian tomato accessions [RCAT030275 (Cegléd), RCAT031012 (Veresegyház), RCAT031095 (Cigánd), RCAT054422 (Jánoshalma)] and a commercial cultivar (Hellfrucht) were investigated according to antioxidant capacity [ferric reducing power (FRAP), total phenol content (TPC) and radical scavenging activity (DPPH)], lycopene and ascorbic acid content. A two year (2013-2014) open field trial was carried out in the certified organic area of SZIE Soroksár Experimental and Educational Station. Our results showed that no significant differences were between the landraces and the variety in DPPH values, while TPC values were higher in both years in the landraces, especially in RCAT031095, and RCAT054422. The lycopene content of RCAT031012, RCAT031095 and RCAT054422 was also higher in both years than those of Hellfrucht. The results demonstrate that small-scale production of the investigated landraces could be marketable.
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KEY WORDS

accession
antioxidant
landrace
lycopene
tomato
vitamin C

Introduction

Tomato is one of the most popular vegetable due to the high number of varieties available, versatile use and advantageous nutritional composition (Tigchelaar 1986). Later it can be regarded as functional food (Jack 1995) as its consumption has a favorable effect on human health (Canene-Adams et al. 2005). Tomato is the most important lycopene source of human, 85% of lycopene consumed originate from tomato (Levy and Sharoni 2004). According to epidemiologic studies, it is suggested that tomato consumption relates to the reduced occurrence of cardiovascular diseases and different cancer types due to carotenoid (Giovannucci 1999) polyphenolic (Vallverdú-Queralt 2012) and vitamin C contents (Adalid et al. 2010).

In the past decades the priority in tomato breeding was to maximize yield, as a consequence, the nutritional values of most modern varieties decreased (Goff and Klee 2006; Klee and Tieman 2013; Tieman et al. 2012). Due to trader and consumer expectations, tomato is harvested in green stage; post-ripened fruits have poor nutritional profile in contrast with those ripened on the vine (Baldwin et al. 2011). To overcome the nutritional loss of modern varieties, several authors suggest reintroducing old varieties and landraces to the production (Male 1999; Rodríguez-Burruezo et al. 2005), in order to enhance nutritional value of novel tomato varieties.

Consumers consider the landraces to be nutritionally richer (Casals et al. 2011) - often without any scientific evidence. Therefore, as a part of a broader study, the aim of this paper is to compare the antioxidant profile of four landraces and a commercial variety for fresh consumption. Except the article by Csambalik et al. (2015), nutritional value of Hungarian tomato landraces was not investigated before.

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Materials and Methods

Plant material

Four Hungarian tomato accessions were selected as appropriate for fresh consumption, three of them are originated from the Central Hungarian Region (Table 1). The landraces were produced together with a control variety, called Hellfrucht (Hild Samen GmbH, Germany) in 2013 and 2014. This commercially available variety gives a round, red, middle-sized

Table 1. RCAT code, origin and fruit characteristics of Hungarian tomato accessions selected for nutritional investigation.

RCAT code	Origin	Year of acquisition	Fruit shape*	Fruit color
RCAT030275	Cegléd	1977	round	orange
RCAT031012	Veresegyház	1987	oblate	red
RCAT031095	Cigánd	1986	flattened	red
RCAT054422	Jánoshalma	2001	elliptic	red

*According to UPOV TG 44/11 Tomato Descriptor

fruit and indeterminate growing habit, similarly to all of the accessions investigated.

The propagation material of landraces was provided by Research Centre for Agrobiodiversity, Tápiószele. The accessions and the variety were produced on the certified organic field of Soroksár Experimental and Educational Station of Szent István University for two years. For the open field propagation, the seedlings were grown in an unheated plastic tunnel both years. The production field was covered by agro textile, under which drip irrigation was established. Each plant was supported by a bamboo pole; side shoots were removed weekly. The production met the standards of organic farming and was free from any chemical treatments. Harvesting was started according to fruit ripening of plants and was conducted weekly. The yield of each accession was measured; the fruits were divided into three fractions: (i) marketable, (ii) cracked, (iii) infected. From the representative sample of each accession, one kg was collected from the marketable group for the measurements. The fruits were free from any visible infection or pest damage. The time of sample collection was adjusted to the maximum weekly yield of each accession and the variety, which was on 14th of August of both years.

Measurements

The samples were chopped and homogenized by a blend mixer after washing at the day of the harvest. The homogenates were filled into Falcon tubes and frozen until the measurements.

The supernatant was collected from homogenates centrifuged at 12 500 rpm and used for antioxidant analyses (FRAP, TPC, and DPPH).

FRAP assay was conducted spectrophotometrically at 593 nm according to Benzie and Strain (1996) with a Hitachi U2900 spectrophotometer. FRAP value was calculated relevant to the activity of ascorbic acid (AA) and expressed as ascorbic acid equivalents. Results were provided in mg AA/l dimension, according to Huang and co-workers (2005). Calibration curve and FRAP reagent were prepared freshly every two hours to avoid influences of AA instability.

The DPPH free radical method was conducted, based on the description of Molyneux and co-workers (2003). From the supernatant, 100 µl was obtained from sample centrifugation and added to 3.9 ml of 6 x 10^{-5} M DPPH solution, kept in the dark for 20 min, then absorbance was recorded at 517 nm with a Unicam Helios Alpha UV-VIS spectrophotometer. Values are shown in percent to the control, where higher values indicate higher antioxidant capacity.

Total phenol content (TPC) was measured using Folin-Ciocalteu's reagent according to the method of Singleton and Rossi (1965). Absorbance was measured at 760 nm with a Hitachi U2900 spectrophotometer and the content of soluble phenols calculated from a standard curve based on gallic acid (GA) concentrations. Results were recorded using mg GA/l dimension.

Ascorbic acid (AA) content was determined by reverse-phase HPLC using an RP-18 column, at 22 °C with a flow speed of 1 L/min. A pH 4.75 buffer made of EDTA and phosphoric acid was used for isocratic elution. Absorbance at 254 nm was measured by UV detector. An extraction solution of 5% phosphoric acid and 0.01% sodium EDTA and a cellulose membrane filter with 0.45 µm pore size were used for sample preparation prior to separation.

Lycopene content was determined with a Unicam Helios Alpha UV-VIS spectrophotometer as described by Fish et al. (2002). Samples were extracted with a solvent containing acetone (with 0.05% BHT), ethanol, and hexane mixture. Absorbance of the extract in the hexane layer was measured at 503nm against hexane as blank solution, and lycopene content was expressed in mg/100 g dimension.

All measurements were performed in triplicate, except for lycopene and DPPH where five replicates were measured. Mean values were compared pairwise with Games-Howell and Tukey post hoc tests. The significance level was 95% ($p<0.05$). All analyses were performed using IMB SPSS Statistics Version 22.

Results

The vitamin C content of investigated accesions and Hellfrucht variety changed between 3.49-8.77 mg/100 g and 2.57-6.22 mg/100 g in 2013 and 2014 (Fig. 1). Regardless to variety, the results of 2014 were significantly lower than those of 2013. In 2013, Hellfrucht, Veresegyház and Jánoshalma significantly differed from Cigánd and Cegléd, while in 2014 there were no significant differences among varieties.

The FRAP values ranged between 17.41-54.47 mg AA/100g and 5.63-21.51 mg AA/100g in 2013 and in 2014, respectively, which differed significantly (Fig. 2). All traits showed significantly lower values in 2014, except those of Jánoshalma. In the first year Veresegyház, while in the second year Hellfrucht gave significantly the highest results.

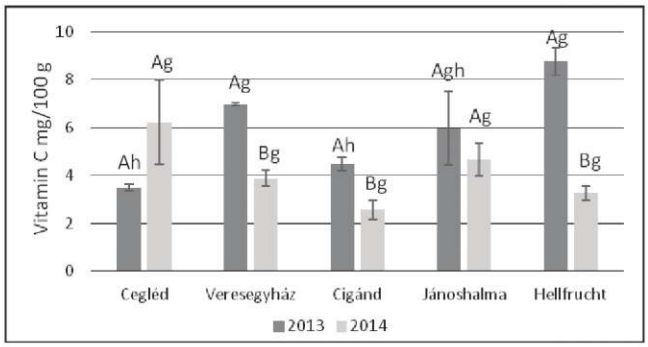

Figure 1. Vitamin C content of tomato accessions and one variety in 2013 and 2014. Capital letters on columns mean significant difference ($p \leq 0.05$) between years of each variety. Lower case letters mean significant difference ($p \leq 0.05$) between varieties within a year.

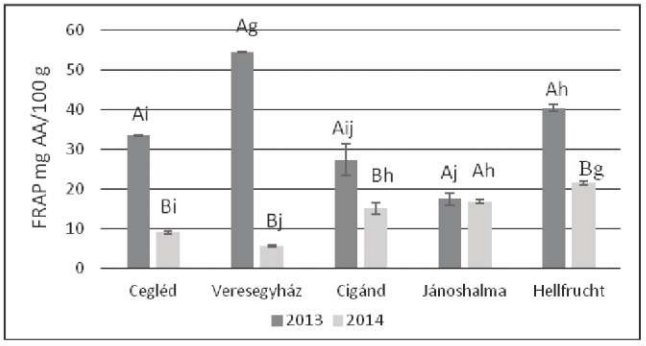

Figure 2. Water-soluble antioxidant power (FRAP) of accessions and one variety in 2013 and 2014. Capital letters on columns mean significant difference ($p \leq 0.05$) between years of each variety. Lower case letters mean significant difference ($p \leq 0.05$) between varieties within a year.

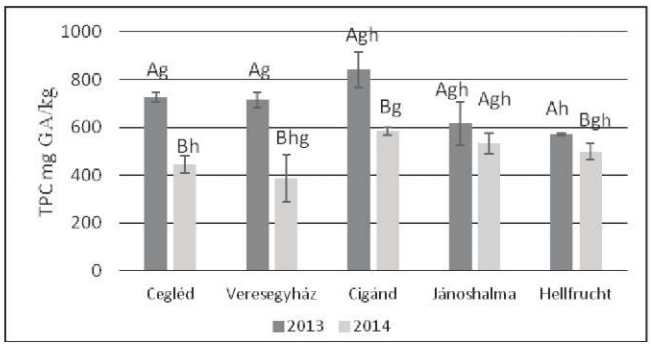

Figure 3. Total Phenolic Content of accessions and one variety in 2013 and 2014. Capital letters on columns mean significant difference ($p \leq 0.05$) between years of each variety. Lower case letters mean significant difference ($p \leq 0.05$) between varieties within a year.

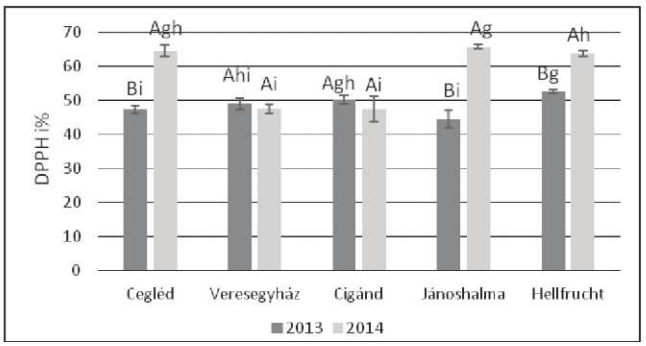

Figure 4. DPPH values of accessions and one variety in 2013 and 2014. Capital letters on columns mean significant difference ($p \leq 0.05$) between years of each variety. Lower case letters mean significant difference ($p \leq 0.05$) between varieties within a year.

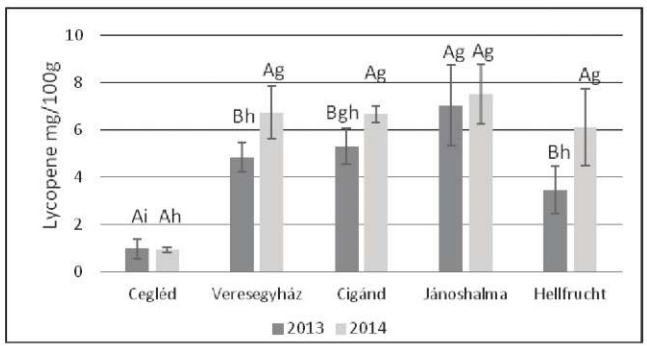

Figure 5. Lycopene content of accessions and one variety in 2013 and 2014. Capital letters on columns mean significant difference ($p \leq 0.05$) between years of each variety. Lower case letters mean significant difference ($p \leq 0.05$) between varieties within a year.

The effect of year was significant on TPC content of investigated accessions and the variety (Fig. 3). The values ranged between 571-841 mg GA/kg and 387-584 mg GA/kg in 2013, and in 2014, respectively. Hellfrucht gave significantly lower results in 2013, while in 2014 only Cigánd differed significantly from Cegléd accession. The TPC of all accesions was higher than Hellfrucht in 2013, Cegléd and Veresegyház differed significantly from the variety. The second year showed lower differences among the samples.

The DPPH values of samples ranged between 44.35-52.59% in 2013 and 47.38-65.72% in 2014 (Fig. 4). The year had no significant influence on the results by Veresegyház and Cigánd. However, in the case of Cegléd, Jánoshalma and Hellfruct, the values of the second year were significantly higher than in the first year. In the first year, Hellfrucht gave significantly the highest results, while Jánoshalma and Cegléd gave significanly lower results. In the second year, Jánoshalma and Cegléd showed the highest results, while Cigánd and Veresegyház gave the lowest ones.

The year had no significant effect on the lycopene content of the investigated samples of Jánoshalma and Cegléd (Fig.

5). However, in case of Veresegyház, Cigánd and Hellfrucht, the values of the first year were significantly lower than those of the second year. In 2013, the values ranged between 3.45-7.02 mg/100 g, while in 2014 it was 6.09-7.48 mg/100 g, except the orange colored Cegléd accession, which had an extremely low lycopene content under 1 mg/100g in both years. The highest results were given in both years by Jánoshalma, differing significantly from Hellfrucht, Veresegyház and Cegléd in 2013. All accessions exceeded the values of the variety in both years.

Table 2. Weather conditions of the experimental site between 1-14 August 2013 and 2014.

Year	Precipitation (mm)	Average minimum temperature (°C)	Average maximum temperature (°C)	Average temperature (°C)
2013	0.8	13.33	31.55	22.65
2014	94.8	16.16	29.72	22.19

Discussion

The results clearly show the influence of weather conditions on the investigated parameters. The vegetation period of 2013 was rather arid with a moderately high average temperature; that of 2014 was rich in precipitation and the temperature was generally lower than in the previous year (Table 2). The statistical analysis showed significant effect of the year on every parameter, except of lycopene. The main environmental factor of lycopene synthesis is temperature (Helyes et al. 2006; Ishida 1999), which showed no significant difference between the two years. Indeed, the time of exposure to sunlight was different in the two years, as the second year took significantly higher amounts of precipitation and the clouds could shade the plants. This effect is demonstrated in the results of vitamin C content. According to the literature (Davies and Hobson 1981; Dumas et al. 2003), vitamin C synthesis strongly correlates with the exposure to sunlight with the exception of Cegléd. All accessions, as well as Hellfrucht gave lower results in the second year. Cegléd accession with orange colored fruits had a different reaction to the theoretically lower exposure to sunlight: vitamin C results could be doubled in the first year.

The effect of increased precipitation is well demonstrated on the FRAP results and a lower extent on TPC results. In both parameters, the results of the second year are lower, due to the 'diluting' effect of heavy rainfall. As 20-30% of FRAP values are given by vitamin C content (Cano et al. 2003), Veresegyház and Hellfrucht rich in ascorbic acid, so it could maximize the FRAP values over the other accessions. FRAP also measures phenolic content as water-soluble antioxidants, and TPC method is not selective to phenolic components, it also reacts with vitamin C (Apak et al. 2007; Balogh et al. 2010; Singleton et al. 1999). In contrast with its low vitamin C content, the relatively high FRAP values of Cegléd accession is supported by its higher TPC results. It can be concluded, that Veresegyház and Hellfrucht are rich in ascorbic acid and phenolic components. Cegléd and Cigánd are characterized rather by high phenolic content and Jánoshalma contains lower amount of both components. To overcome the selectiveness of antioxidant measurement methods, the application of three or more methods is suggested (Hegedűs et al. 2010; Huang et al. 2015). According to literature, including our previous experiences (Csambalik et al. 2015) the summarized discussion of the applied antioxidant capacity measurement methods is reasonable. Both analyses indicated the relatively higher antioxidant capacity of Veresegyház and Hellfrucht in 2013. The outstanding TPC result of Cegléd and Cigánd was not supported by the other two methodologies. In this year, TPC was rather an outlying factor; this might be due to the higher average temperature, which supports the synthesis of phenolic components (Rivero et al. 2001). In 2014 FRAP was the method, which showed gradually lower values, but these results were generally in coherence with the other two methodologies. The weather conditions of the second year were favorable for Cigánd, Jánoshalma and Hellfrucht from the viewpoint of antioxidant capacity. In contrast with the high DPPH values of Cegléd, FRAP and TPC results did not support the high antioxidant power of that accession.

Being a fat-soluble antioxidant, lycopene levels of samples does not influence water-soluble antioxidant measurement results. However, significant differences exist between the samples for that trait. As was expected, due to its color, Cegléd accession is poor in lycopene. The moderately lower average temperature of the second year had a favorable impact on the lycopene levels, although this effect was not significant in case of all the samples. This might be due to the relatively low variance of temperature over the two years. In both years, Jánoshalma accession gave the highest results, in contrast with its rather low water-soluble antioxidant power. This means, that lycopene could be the main advantage of this accession. With the exception of Cegléd, all accessions had higher lycopene content than Hellfrucht, in both years. This could be a genetically encoded advantage of accessions over commercial varieties.

Conclusion

Landraces are considered as old varieties with lower yield quality and quantity, but with more favorable nutritional value, in contrast with modern commercial varieties and hy-

brids. Our study aimed to investigate the antioxidant profile of four Hungarian tomato accessions for fresh consumption and to compare the results with a commercial variety. The results showed, that no general statements can be drawn as differences among the investigated accessions exist. The comparison of accessions and the variety showed that no great differences are given between the two groups. Hellfrucht variety can be characterize by relatively high ascorbic acid content, by a moderately high antioxidant power and by low lycopene content among investigated accessions. From the point of water-soluble antioxidant power, Veresegyház and Cigánd are promising, while Jánoshalma is valuable due to its high lycopene content. After investigating the yield characters of these accessions, small-scale production could be marketable.

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ARTICLE

Bioanthropological analysis of Békés 103 (Jégvermi-kert, Lipcsei-tanya), a Bronze Age cemetery from southeastern Hungary

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ABSTRACT The analysis of human skeletal remains is an important tool for reconstructing aspects of health, diet, disease, and population structure (such as demographics, life expectancy, and mortality) in past populations. This study presents the recovery techniques and initial bioanthropological analysis of cremation and inhumation burials from the archaeological site of Békés 103, a Bronze Age cemetery located in southeastern Hungary. In all possible cases, analysts microexcavated cremation urns in a laboratory setting to ensure recovery of all fragmentary skeletal material and allow detailed spatial documentation of each burial's contents. This report presents estimated age and sex information for each individual, provides a preliminary assessment of observable trends in the population, and offers a paleodemographic profile of the cemetery for comparison with contemporary prehistoric communities in Hungary.
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KEY WORDS
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Introduction

Despite a long history of archaeological research in the Lower Körös Basin (Körös-vidék) of southeastern Hungary, we know little about the mortuary practices and population structure of people living there during the Bronze Age (2700-900 cal BC). This absence is finally being corrected through fieldwork at the Békés 103 (Jégvermi-kert, Lipcsei-tanya) site, and analysis by the Bronze Age Körös Off-Tell Archaeology (BAKOTA) project reported here provides a first account of the mortuary and biological characteristics of the population in this region. The recovered skeletal material is primarily cremation, and though comparison with other cremation traditions would be ideal, there are no detailed publications of bioanthropological data for cremation cemeteries nearby. In this report, we therefore present age and sex data for all excavated burials to date, offer a paleodemographic profile for the cemetery during the most intensive 300 years of use, and provide a comparison of the burial and demographic patterns with data from two neighbouring culture groups, the Maros and Füzesabony.

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Archaeological background

The Békés 103 (Jégvermi-kert, Lipcsei-tanya) site is situated in the Lower Körös Basin on an ancient meander of the Kettős-Körös River, near the modern town of Békés (Fig. 1). Hungarian archaeologists discovered the multicomponent site through systematic field walking surveys in the 1990s, and discovery of burned human bone and Bronze Age urn fragments in the early 2000s revealed that the area had been used as a cemetery (Duffy et al. 2014).

The BAKOTA project carried out archaeological fieldwork at the Békés 103 site between 2011 and 2015. Over four field seasons, excavation revealed human remains in 67 of the 68 identified human burial (HB) features, and cremation of human bones makes up the dominant body treatment (91.2% of burials). In fifty-eight cases, mourners placed the cremains in a ceramic funerary urn (Fig. 2), but in two occasions practiced scattered cremation (Fig. 2). Five graves contained inhumations (Fig. 2), and in three cases, the precise burial rite was unclear, though two of these contained cremated human bone.

Our stylistic analysis and radiocarbon dating of the burials at Békés 103 suggest ancient people used the site as a burial ground from the Early to Late Bronze Age. However, the majority of the graves fall within a much narrower timeframe,

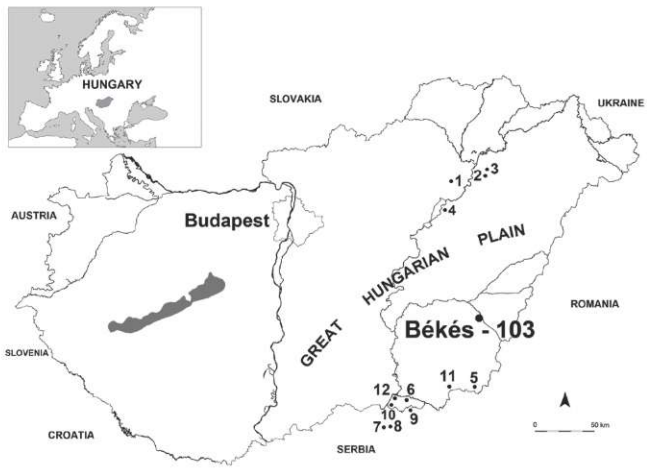

Figure 1. The geographical location of the Bronze Age Békés 103 site and Bronze Age sites used for comparison (Füzesabony group: Gelej-Kanális-dűlő (1), Polgár-Homok-dűlő (2), Polgár-Kenderföldek - Majoros-tanya (3), Tiszafüred - Majoroshalom B, D (4); Maros group: Battonya-Vörös Október TSZ (5), Deszk A,F, (6), Mokrin (7), Ostojicevo (8), Óbéba (9), Ószentiván (10), Pitvaros (11), Szőreg C (12).

between 1600 and 1300 cal BC. Most researchers generally consider this time range to represent the final centuries of the Middle Bronze Age (2000 and 1450 cal BC) and the early phase of the Late Bronze Age in the Carpathian Basin (Fischl et al. 2013; Fischl et al. 2015; Kiss et al. 2015).

The culture groups used for comparison to the population of Békés 103 overlap in time with some, but not all, of the cemetery's period of most intensive use (O'Shea 1996; O'Shea et al. 2011; Fischl et al. 2013; Fischl et al. 2015). In contrast with the Békés 103 population, both the Maros and Füzesabony groups practiced inhumation as a normative body treatment (O'Shea 1996; Csányi 2003), though cremation becomes common in the later phase of Füzesabony (Polla 1960; Fischl 1999). Yet these groups still serve as the best points of comparison with the Körös Basin cemetery. Like the community at Békés 103, Maros and Füzesabony groups also inhabited the Eastern Carpathian Basin during the Middle Bronze Age (see Fig. 1 for site locations referenced in this study), and all three groups share common ceramic styles (such as the swedish helmet bowl), technological traditions (Kreiter 2005), and settlement structures (Bóna 1993). Despite the cultural and temporal differences, therefore, these two groups will serve as a first line of comparison to highlight obvious digressions in mortuary behaviour and demography.

Methods

Microexcavation

Field workers excavated inhumations and scattered cremations in situ, and photographed and mapped all burials in the field during excavation, but we transported cremations in funerary urns to the lab to be carefully microexcavated in a controlled setting. Where possible, we used layer-by-layer microstratigraphic excavations to recover fragmentary skeletal material, but where natural stratigraphy was absent, we removed urn fill in arbitrary 2-4 cm levels. We photographed each level and recorded observations about that levels' characteristics on a detailed profile map (Fig. 3). This included descriptions of the colour and texture of the urn fill for each context, approximate thickness of the layer, the presence of non-skeletal material, and the location of samples taken for further analysis (such as ancient DNA and isotopic analysis). When visible, excavators recorded the position and direction of fragments or bone clusters, the spatial distribution of the bones by anatomical regions, and morphological or metric data indicative of the individual's age at death or sex. We also noted preliminary observations about heat-related color and/or fracture changes due to the cremation process, and pathological alterations with their possible etiology. We assigned a discrete identification number to bones from each

Figure 2. Body treatment at the Békés 103 cemetery: urn cremation (HB 06) (a), scattered cremation (HB 04) (b), inhumation (HB 03) (c). Photo credits: Paul R. Duffy.

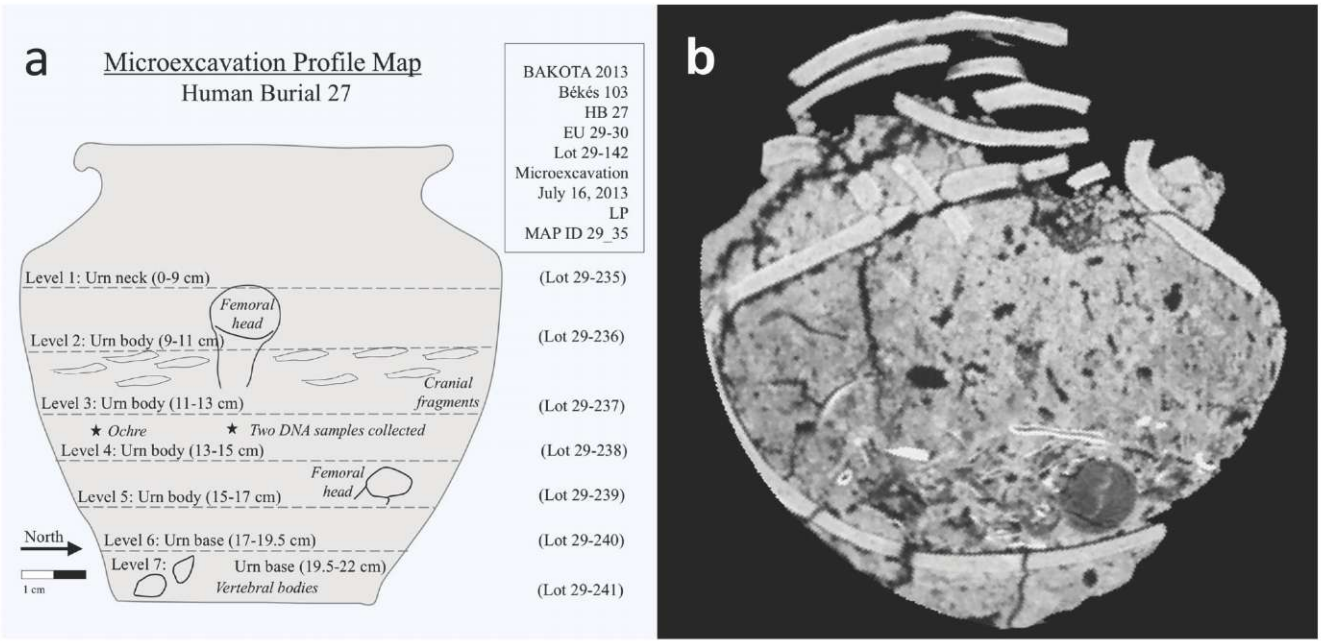

Figure 3. Digitized and simplified microexcavation profile map of HB 27 (a). CT image of HB 27 showing a measurable femoral head (dark grey circle on bottom right) (b).

level, and then washed, counted, and weighed them according to context to maintain the spatial resolution of the dataset for future analyses.

Computed tomography

We scanned ten cremation urns prior to microexcavation using computed tomography (CT). Scans were conducted on a GE Lightspeed unit, using a 140 kV at 400 mA exposure with a slice thickness of 0.625 mm. The scans provided a non-destructive technique to explore the internal composition of the urns prior to the removal of the burial contents, and CT images were useful for documenting the position, density, and relative stratigraphy of skeletal material and artifacts within the urn (Fig. 3). In a few cases, we used these images to measure critical elements of intact bones before removal (such as the diameter of a delicate unfused femoral head of a Juvenis individual featured in Fig. 3), improving chances of assigning age at death.

Age and sex estimation

We estimated age at death for subadult individuals using known patterns of bone growth and development, including morphological and metric characteristics of juvenile bones (Fazekas and Kósa 1978; Stloukal and Hanáková 1978 Schaefer, Black and Scheuer 2009), and the sequence of tooth formation and eruption (Ubelaker 1989; Smith 1991). Our analysis estimated adult age based on morphological changes in the pubic symphysis (Suchey and Katz 1998), and age-dependent changes in rib and clavicle ends (Loth and Iscan 1989). We categorized the results of the age estimation using Martin's classification (Martin and Saller 1957). In cases, where poor preservation or the incompleteness of skeletal material prevented attribution to a Martin classification, we used intermediate (*e.g.*, Inf I - Inf II) or even broader categories (*e.g.*, subadult, adult) (see Results).

The biological sex of adults was based on anatomical sites/areas showing sexual differences. Using metric and non-metric characteristics of both the skull and postcranial skeleton, we scored twelve and nine anatomical areas, respectively (Éry et al. 1963). We characterized individuals as "indeterminate" in cases where no age or sex-related morphological or developmental traits were observable.

Life expectancy and mortality calculations

We used physiological age determinations to estimate life expectancies and mortality characteristics for the population. Life tables allow calculation of survival probability in a population for a given age category, which can then be used for comparison to other populations or modeling of living population size (Acsádi and Nemeskéri 1970). This calculation makes several assumptions (*i.e.* the population is stable, that death rates are uniformly distributed across age and sex categories, and that the archaeological population accurately

Table 1. Determination of age at death, sex, and number of individuals in each human burial. Key: Burial no: HB (human burial); Body treatment: UC (urn cremation), SC (scattered cremation), I (inhumation); Age: age in weeks refers to prenatal life; Sex: M (male), F (female), I (indeterminate sex).

Burial no.	Treatment	Age category	Age	Sex	Burial no.	Treatment	Age category	Age	Sex
HB 01	UC	Juvenis	13-18 ys	I	HB 34	UC	Infant II	6-12 ys	I
HB 02	UC	adult	20-x	M?	HB 35	UC	Inf I - Inf II	1-12 ys	I
HB 03	I	Juvenis	12-14 ys	F?	HB 36	UC	Infant II	6-8 ys	I
HB 03	I	foetus/newborn	week 40	I	HB 37	UC	adult	20-25 ys	F
HB 04	SC	adult	20-x ys	I	HB 38	UC	Inf I - Inf II	1-12 ys	I
HB 05	UC	indeterminate	-	I	HB 39	UC	subadult	1-19 ys	I
HB 06	UC	adult	20-x ys	F	HB 40	UC	adult	20-x ys	M
HB 07	UC	adult	20-x ys	I	HB 41	UC	Adultus	25-39 ys	F
HB 08	UC	Adultus	21-25 ys	F	HB 42	UC	Infant I	2-3 ys	I
HB 08	UC	Infant I	1-6 ys	I	HB 43	I	Infant I	0-0.5 ys	I
HB 09	UC	Adultus	20-40 ys	F	HB 44	I	foetus/newborn	week 32-36	I
HB 10	UC	Ad-Mat	30-49 ys	F?	HB 45	UC	adult	20-x ys	M
HB 11	UC	Infant I	2-6 ys	I	HB 46	UC	adult	20-x ys	M
HB 12	UC	subadult	0-19 ys	I	HB 47	UC	Ad-Mat	35-50 ys	F
HB 13	UC	foetus/newborn	week 36-40	I	HB 48	UC	Adultus	25-39 ys	F
HB 14	UC	Infant I	3-5 ys	I	HB 49	UC	adult	20-x ys	I
HB 14	UC	Adultus	20-25 ys	I	HB 50	UC	Inf I - Inf II	1-12 ys	I
HB 15	UC	Infant I	4-6 ys	I	HB 51	unknown	indeterminate	-	I
HB 15	UC	adult	20-x ys	I	HB 52	I	foetus/newborn	week 38	I
HB 16	UC	adult	20-x ys	I	HB 53	UC	adult	20-x	I
HB 17	UC	Juvenis	16-20 ys	F?	HB 54	UC	adult	20-x ys	M
HB 18	UC	Infant I	1-3 ys	I	HB 55	SC	Infant I	1-2 ys	I
HB 18	UC	adult	20-x ys	F	HB 56	UC or SC	Inf I - Inf II	1-12 ys	I
HB 19	UC	indeterminate	-	I	HB 57	UC	Inf I - Inf II	1-12 ys	I
HB 20	UC	Adultus	30-40 ys	I	HB 58	UC	subadult	1-19 ys	I
HB 21	UC	adult	20-x	F	HB 59	I	Infant I	1-6 ys	I
HB 22	UC	Infant II	6-12 ys	I	HB 60	UC	Ad-Mat	35-50 ys	I
HB 23	UC	Infant I	1-6 ys	I	HB 61	UC	Infant I	1-6 ys	I
HB 24	UC	Adultus	20-24 ys	I	HB 62	UC	Maturus	40-59 ys	F
HB 25	unknown	no remains	-	-	HB 62	UC	foetus/newborn	week 30-32	I
HB 26	UC	Infant I	1-3 ys	I	HB 63	UC	Inf I - Inf II	1-12 ys	I
HB 27	UC	Juvenis	13-19 ys	I	HB 64	UC	Infant I	1-6 ys	I
HB 28	UC	Adultus	25-35 ys	F	HB 65	UC	Infant I	1-6 ys	I
HB 29	UC	adult	20-x ys	I	HB 66	UC	Maturus	40-59 ys	M
HB 30	UC	adult	20-x ys	I	HB 66	UC	Inf II - Juv	6-19 ys	I
HB 31	UC	Infant II	6-12 ys	I	HB 67	UC	adult	20-x ys	I
HB 32	same as HB 39				HB 68	UC	indeterminate	-	I
HB 33	UC	indeterminate	-	I	HB 69	UC	adult	20-x ys	F

represents the true biological population). In this study, the cemetery population spans several generations, which makes the assumption of stationarity a reasonable one to make (Sattenspiel and Harpending 1983). In every possible case, we carefully microexcavated the cremation urns in the lab ensuring recovery of very small skeletal remains (easily missed or misidentified in field settings). Life expectancy values were calculated using Bernert's (2005) anthropological programme pack, based on Acsádi and Nemeskéri (1970). Due to the difficulty of making sex determinations on cremated bone, we excluded sex data from our estimation of life expectancy, and assumed a balanced distribution of sexes among adults. By convention, the highest estimated age within a cemetery is used as a maximum lifespan for that cemetery, so in calculating life expectancy at Békés 103, we used 60 years as the maximum lifespan (Table 1). This baseline lifespan maximum obviously has implications for comparing cemeteries with greatly different sample sizes or different body treatments, where cultural practices (such as cremation) preclude confident identification of much older individuals. We address these concerns in the discussion.

It is also important to note that, according to radiocarbon dates and a preliminary stylistic analysis of the ceramics, six of the 67 human burials with human remains belong to chronological periods earlier or later than the majority of the cemetery's population. For this reason, we exclude the individuals in these burials (HB 21, HB 54, HB 55, HB 57, HB 59, and HB 62) from the paleodemographic analysis in

order to ensure a representative population for the timeframe between 1600 and 1300 cal BC.

Results

We analysed human bone fragments from 67 graves and identified skeletal remains for 74 individuals (Table 1).

Estimated number of individuals

Ninety percent of the graves contained bone fragments belonging to a single person, but in the case of one inhumation (HB 03) and six urn cremations (HB 08, HB 14, HB 15, HB 18, HB 62, and HB 66) we identified two individuals (an adult and a subadult in a majority of cases) during the osteological analysis. In most cases, a single ceramic vessel included the bones of both individuals; the second person's remains formed single or multiple clusters of bones within the osseous matrix of the first individual. However, HB 08 represents another type of double burial. In this case, the mourners placed two adjoining cremation urns in a single pit. Interestingly, both vessels included fragments of both individuals, but one of the urns contained mostly adult bone fragments, while the other vessels contained a majority of subadult pieces. In two other urns, we identified a single fragment of another individual included with the primary skeletal contents. In these cases, it was not possible to determine the age/sex of the extra skeletal material or even whether the burial was an intentional double burial. We therefore did not count these remains as separate individuals at this stage of the analysis. Future investigations will explore whether patterns of single bone inclusions reflect actual mortuary practices or simply secondary byproducts of some other behavior. HB 03 is the only inhumation that included skeletal remains of two individuals: a fairly well preserved 12-14 year old and fragments of a foetus/newborn.

Three burials (HB 19, HB 25, HB 32) require further explanation with respect to identification of individuals in the cemetery. In the case of HB 19, we collected a few bones during excavation, but most remains were left unexcavated because we identified the burial at the edge of a profile wall that extended into a contemporary road, making extension of the excavation unit impossible. Our conservative preliminary estimate is that this burial represents a single individual. We identified HB 25 by the base of a small vessel usually included as a grave good along with a cremation urn or an inhumation; however, the area around the vessel was highly disturbed and no human remains were found during the excavation. While this feature may represent a burial of some kind regardless of the presence of skeletal material (*e.g.*, symbolic), due to preservation, we cannot be sure an individual was included. Finally, we initially designated HB 32 as a distinct, and highly disturbed, cremation urn burial located next to another burial, HB 39. Analysis of the skeletal and ceramic data post-excavation revealed that the remains were from the same burial, so we combined the remains for further analysis under the designation HB 39.

Paleodemographic profile

The following estimates of population structure exclude six chronological outlier graves (and seven individuals), and include only the 61 burials (and 67 individuals) that were buried at the site between 1600 and 1300 cal BC. From these we could determine the age for 62 individuals. Concerning the distribution of age at death, subadults and adults are balanced with 29 and 33 people belonging to these broad age categories, respectively (Fig. 4). All of the subadult age categories are present, but the Infant I category is the most represented (12/33). The adultus age category (20-39 years) is the most represented sub-category among adults (8/29). The prevalence of individuals in older age categories decreases with age, though we were not able to determine the precise age of 17 adults.

Determination of biological sex is difficult in cremated human remains due to fragmentation and the absence of diagnostic morphological characteristics. In this sample, we were able to determine the biological sex in 48.3% of adults, if we also include likely sex designations (*i.e.* M?). We identified ten females and four males (Fig. 4).

The table below illustrates the calculated life expectancy for the Békés 103 cemetery at different age intervals assuming a maximum life span of 60 years. Life expectancy at birth was 21.88 years, while those who survived to early adulthood (20-24 years) were expected to live another 17.47 years (Table 2).

We assumed a balanced sex ratio and did not calculate mortality curves by sex due to the fragmentary nature of the cremated remains and the limited number of sex determinations. The cemetery's mortality profile has three peaks (Fig. 5). The highest and most characteristic one appears between 1 and 4 years of age, and it is followed by a rapid, monotonic decline to a low point at around 15-19 years of age. The second peak is at early adulthood (20-24 years), while a less pronounced peak is also recognizable between 35-39 years of age. The mortality curve presents a slow decline at older age categories, but the decline is not monotonic.

Discussion and Conclusion

The bioanthropological analysis of the Békés 103 cemetery provides the first dataset of its kind for the Lower Körös

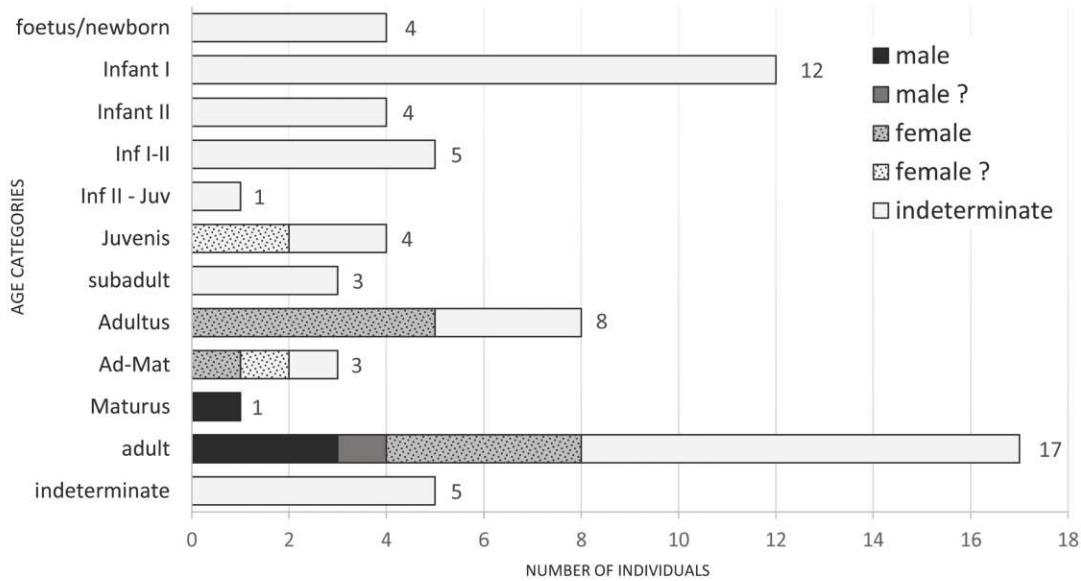

Figure 4. Distribution of age at death and biological sex in the Békés 103 cemetery.

Basin in southeastern Hungary. Below, we offer preliminary comparisons to previously published skeletal collections found in the Maros and Füzesabony Bronze Age cultural traditions from the eastern Carpathian Basin (for site locations mentioned in the text, see Fig. 1).

Seven burials from this study contained the remains of more than one individual. The majority of these burials included an adult (both males and females were identified) and the remains of a subadult under age six. Exceptions include one cremation urn (HB 66) that contained bones of an adult and an Infant II-Juvenis (6-19 years), and an inhumation (HB 03) that contained the skeletal remains of a subadult (12-14 years old) and a foetus-newborn.

Burying multiple individuals in a single grave is not uncommon during the Bronze Age in the eastern Carpathian Basin, but researchers identified only a few cases in cemeteries from Maros and Füzesabony groups (Hajdu 2012; O'Shea 1996). These cases include the Mokrin and Szőreg C cemeteries in the Maros area, where both male and female adults are buried with subadults, both infants and children (O'Shea 1996). The relationship between the individuals in double burials is not known, though parent/child relationships, and expedient practical parameters (for example, similar timing of death) may be responsible for the pairing. Reports from Füzesabony cemeteries Polgár-Kenderföldek-Majoros-tanya, Tiszafüred-Majoroshalom (B, D), and Gelej-Kanális-dűlő, Polgár-Homok-dűlő, note burials with multiple individuals with a wider variety of age combinations (subadult-subadult, subadult-adult, and adult-adult combinations) and a diverse sex distribution among them (Hajdu 2012; Zoffmann 2006).

Table 2. Life table of the Békés 103 cemetery (estimated maximum life span = 60 years). Dx: death's no, dx: percentage of deaths, lx: survivors entering, qx: probability of death, Lx: total years lived between x and x + 5, Tx: total years after lifetime, ex: life expectancy.

Age groups	Dx	dx	lx	qx	Lx	Tx	ex
0	4,0	5,97	100,00	0,04	97,01	2188,08	21,88
1-4	11,6	17,26	94,03	0,12	341,60	2091,07	22,24
5-9	9,4	13,96	76,77	0,12	348,93	1749,47	22,79
10-14	5,7	8,53	62,80	0,09	292,69	1400,54	22,30
15-19	3,7	5,59	54,27	0,07	257,38	1107,85	20,41
20-24	6,3	9,37	48,68	0,13	219,99	850,47	17,47
25-29	4,3	6,36	39,31	0,11	180,67	630,48	16,04
30-34	4,4	6,62	32,95	0,13	148,21	449,81	13,65
35-39	4,7	7,01	26,33	0,18	114,14	301,61	11,45
40-44	3,6	5,42	19,32	0,19	83,05	187,47	9,70
45-49	3,5	5,22	13,90	0,25	56,45	104,41	7,51
50-54	2,7	4,10	8,68	0,32	33,16	47,97	5,53
55-59	2,6	3,91	4,58	0,57	13,13	14,81	3,23
60-64	0,4	0,67	0,67	0,67	1,68	1,68	2,50
65-69	0,0	0,00	0,00	0,00	0,00	0,00	0,00
70-74	0,0	0,00	0,00	0,00	0,00	0,00	0,00
75-79	0,0	0,00	0,00	0,00	0,00	0,00	0,00
80-84	0,0	0,00	0,00	0,00	0,00	0,00	0,00
85-89	0,0	0,00	0,00	0,00	0,00	0,00	0,00
90-94	0,0	0,00	0,00	0,00	0,00	0,00	0,00
95-99	0,0	0,00	0,00	0,00	0,00	0,00	0,00
Total	67,0	100,00					

The age distribution of the Békés 103 cemetery is similar to Füzesabony group cemeteries in that all age categories can be found there (Zoffmann 2006, 2011; Hajdu 2012). This

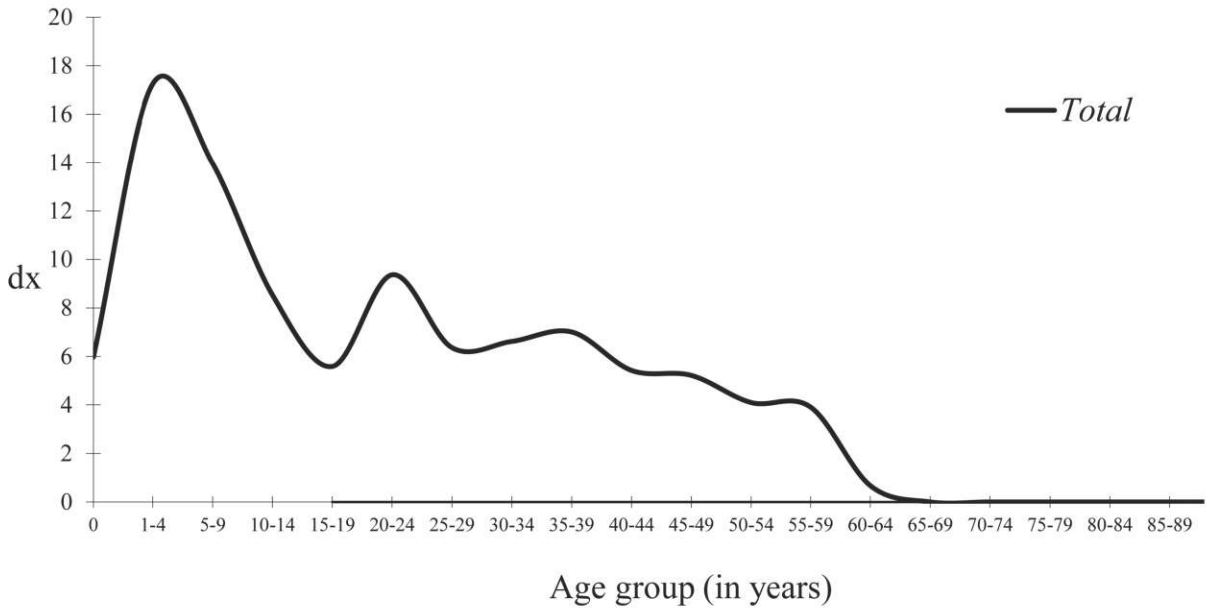

Figure 5. Mortality profile of the population of Békés 103 cemetery (estimated maximum life span = 60 years).

varies from what has been documented at most Maros cemeteries (*e.g.*, Deszk A, Deszk F, Mokrin, Ószentiván, Óbéba, Pitvaros), where young infants under three years of age are missing from the cemetery population. This absence suggests an alternate funerary custom for the very young, and settlement excavation has identified infant burial (O'Shea 1996). Subadult grave pits are significantly shallower than adults at Deszk F, Mokrin, Pitvaros and Szőreg C however, so taphonomic processes such as erosion cannot be excluded as the source of missing very young individuals (O'Shea 1996). For this reason, the cemetery of Ostojićevo is rare in the Maros for containing the full range of age categories (Zoffmann 2006, 2011; Hajdu 2012).

Accurately determining biological sex in a mostly cremated sample is difficult due to the fragmentary nature of burned human bone, so the cremains from Békés 103 include a relatively small number of positive identifications. Nonetheless, the sex data for adults in the cemetery are quite disproportionate, with females more than doubling the number of identified males. It is worth noting that an imbalanced sex distribution is common from several nearby Bronze Age cemeteries of the Maros (*e.g.*, Deszk A, Mokrin, Ószentiván, Szőreg) and Füzesabony (Gelej-Kanális-dűlő, Polgár-Kenderföldek-Majoros-tanya) (Farkas and Lipták 1971; Farkas 1975; O'Shea 1996; Zoffmann 2006, 2011; Hajdu 2012). The cause of the distribution is not known, though O'Shea suggests that the participation of males in warfare away from home plausibly accounts for it (O'Shea 1996). While it is interesting that we also found a female predominance in our sample, a larger sample size would be needed to demonstrate its significance.

We present the calculated life expectancy at birth for Békés 103 alongside published Maros and Füzesabony values in Figure 6. Though Békés 103 is the lowest of the bunch, there are two important factors influencing this pattern. First, because identification of older individuals at Békés 103 is difficult due to the cremation, the upper age limit used for the cemetery (60 years) may be more an artifact of sample size and the vagaries of the cremains than the actual maximum age of the population, though older individuals are sometimes identified in cremation cemeteries of similar sample size (Zoffmann 2011). At inhumation cemeteries, however, individuals are routinely identified as 70 or 75 years of age, and setting the age maximum 10-15 years higher at Békés 103 would raise the life expectancy at birth by 2-3 years. Second, most of the inhumation cemeteries in the Maros areas lack the youngest individuals (see above), resulting in higher life expectancies at birth than normal. Without correction by addition of infants to the dataset, these values are not directly comparable to populations with young individuals present (for an example of a correction, see O'Shea 1996). Besides these methodological factors, diet, pathological conditions, and way of life could also affect life expectancy. In the future, correction of datasets missing young infants, and inclusion of cremation cemeteries with similar age maximums, will allow meaningful comparison of life expectancies at birth. As it currently stands in Figure 6, it is clear that the life expectancy values of Maros cemeteries are higher than values at Füzesa-

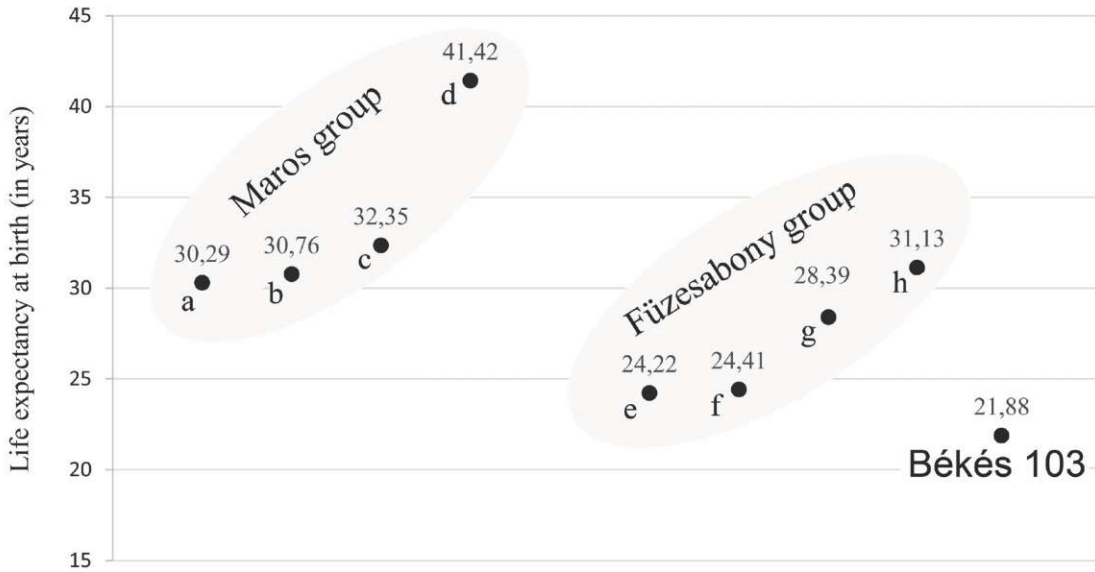

Figure 6. Life expectancy at birth values from Bronze Age cemeteries. Maros group: Battonya-Vörös Október TSZ (Zoffmann 2011) (a), Ostojicevo (Zoffmann 2011) (b), Mokrin (Zoffmann 2011) (c), Szőreg C (Zoffmann 2011) (d). Füzesabony group: Polgár- Kenderföldek - Majoros-tanya (Zoffmann 2011) (e), Tiszafüred - Majoroshalom B, D (Hajdu 2012) (f), Gelej-Kanális-dűlő (Hajdu 2012) (g), Polgár-Homok-dűlő (Zoffmann 2011) (h).

bony sites and Békés 103 because the youngest individuals in the population are not present in the cemeteries at the former, but are present in the latter.

Researchers identify a U-shaped pattern of age-specific mortality across a wide range of human populations, indicating the highest mortality for the very young and old (Wood et al. 2002). Our sample only partially fits this profile. The high mortality values in the first few years of life in the Békés 103 sample might be associated with higher susceptibility to factors such as infections or other pathological conditions. This is a typical trend found in prehistoric populations, and consistent with naturally-occurring biological expectations (Rega 1997; Hoppa 2002). Our sample lacks the second high mortality peak for the older age category characteristic of most human populations. It is very difficult or impossible to identify subtle age related changes in the cremated bone of older individuals, possibly making the right end of the mortality profile misleading. A larger dataset may help clarify whether this pattern actually reflects some biological reality, or simply the limitations of our dataset.

Summarizing our results, we can say that the basic paleodemographic data provided by classical bioanthropological methods and medical imaging techniques give us useful information for the Bronze Age Békés 103 cemetery's population structure. The preliminary results of Bronze Age skeletal material at Békés 103 provide both similarities and contrasts with other Bronze Age populations, and offer several avenues for future explorations. These data form part of several ongoing BAKOTA research projects focused on understanding mortuary customs at the Békés 103 site. Future studies will also incorporate these data into the analysis of bone weight, heat-related color and fracture changes, the spatial distribution of bones in urns, the chemical composition of bone and ceramics, and the stylistic and spatial characteristics of associated grave goods.

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